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# **The significance of genetic diversity of *Blastocystis* in different hosts**

**Thesis submitted for the degree of doctor of philosophy**

**By**

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## Abstract

*Blastocystis* is an obligate anaerobic, protistan parasite found in the intestinal tract of human and various other hosts. *Blastocystis* is placed within the stramenopiles. This diverse group also includes slime nets, water moulds and brown algae. The transmission of *Blastocystis* is believed to take place through the faecal–oral route. Waterborne transmission of *Blastocystis* through the use of untreated water or poor sanitary conditions has also been reported.

Numerous epidemiological surveys have shown prevalence up to 10% of the population in developed countries and as much as 50–60% in developing countries. Differences in virulence among *Blastocystis* subtypes have been reported in a recent animal infection study. *Blastocystis* shows extensive genetic diversity and is divided into numerous genetic subtypes

The parasite is commonly associated with gastrointestinal symptoms such as watery and mucous diarrhea, vomiting, and abdominal cramps and bloating. Epidemiological studies suggest an association between *Blastocystis* infection and irritable bowel syndrome.

Irritable bowel syndrome (IBS) is identified as a functional bowel disorder in which abdominal pain or discomfort is associated with a defect or alteration in the consistency or frequency of stools. Diagnosis of IBS by physicians is carried out using symptom –based criteria known as the Rome criteria. To see whether there is any link between *Blastocystis* infection and irritable bowel syndrome (IBS), I have compared the frequency of subtypes of *Blastocystis* in IBS patients with those in the general population.

In human population both UK and Libya showed similar distribution of *Blastocystis* subtypes

ST 1, 2 and 3 are common in the two populations and ST3 has the highest frequency in UK while ST1 was the most common in Libya.

Epidemiology studies on *Blastocystis* infection in animals have revealed high frequency of occurrence in cattle, pigs, primates and birds and it has often been suggested that *Blastocystis* infection is a zoonosis. In Libya, *Blastocystis* subtypes were detected from humans, birds and numerous mammals' hosts (camel, cow, sheep, goat, gazelle, Barbary sheep and gundi). Ten subtypes were detected (1, 2, 3, 5, 7, 10, 14, 15, 16, 17) and four new subtypes were found in cow, camel and gundi. Subtype 1, 3, and 7 were in common between animal and human but subtypes 5, 10 and the four new subtypes were found only in animals. ST 2 was found in human only.

Also I discovered four new hosts for *Blastocystis* from mouse deer, gundi, gazelle and barbary sheep. Both human and animal showed diversity in *Blastocystis* subtypes.

Both human and animals become infected with same *Blastocystis* subtype and for this reason we need to find refined tool to differentiate between them, so I have developed MLST for *Blastocystis* subtype 1 based on mitochondrial DNA. Application of MLST to 39 isolates from different host and different geographic place showed variation in the sequence of related isolates. Over all MLST proved to be a highly discriminatory and stable method for unambiguous characterization of *Blastocystis*.



## **Declaration**

I declare that all of the work included in this thesis is my own work unless otherwise stated.

All PCR and sequences were performed at London School of Hygiene and Tropical Medicine (LSHTM). All culture of Libyan samples from humans and animals were carried out in Sebha Central Medical Laboratory in Libya. All microscopic images were captured by me with assistance from Mr John Williams in Diagnostic Parasitology Laboratory of the London School of Hygiene and Tropical Medicine.

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## **CHAPTER 1**

### **Introduction**

## 1.1 History

*Blastocystis* is an intestinal parasite of humans and various other hosts. In 1911, Alexeieff (Zierdt, 1991a) was the first to describe the genus *Blastocystis* and proposed the name *Blastocystis enterocola*. Later, the organism was designated as a yeast by Brumpt, in 1912, who gave it the name *Blastocystis hominis* (Zierdt, 1991a). The name *Blastocystis hominis* is now firmly established and recognised worldwide.

After being ignored for decades, this parasite has been recapturing the interest of parasitologists since the work of Zierdt *et al.* (1967). Even though a large number of studies have been performed, the possible pathogenicity of this parasite is still controversial (Haresh *et al.*, 1999, Kuo *et al.*, 2008, Upcroft *et al.*, 1989, Vogelberg *et al.*, 2010, Yakoob *et al.*, 2004). In this work, *Blastocystis hominis* will be referred to as *Blastocystis* sp. or simply by the genus name *Blastocystis* for reasons that will be made clear later.

## 1.2 Taxonomy

The taxonomic status of *Blastocystis* has been an enigma until recently and the history of the organism shows the difficulty in identifying its taxonomic position. Zierdt *et al.* (1967) were the first to provide an ultrastructural study of *Blastocystis* and showed that it was not a yeast or a fungus as suggested before by Alexeieff (1911), Brumpt (1912), Beaurepair Aragoao (1922), Knowles and Das Gupta (1924) and Lavier (1952), nor the cyst of another organism such as a *Trichomonas* sp., as proposed by Bensen (1909) and Bohne and Prowazek (1908). Others described it as vegetable material (cited in Zierdt *et al.*, 1967). Zierdt's conclusion that



it was a protozoan was supported by later ultrastructure studies done by different authors (Boreham and Stenzel, 1993, Dunn *et al.*, 1989, Zierdt, 1991a).

The *Blastocystis* cell reveals protistan features of morphology as it lacks a cell wall but contains one or more nuclei, smooth and rough endoplasmic reticulum, a Golgi complex and mitochondrion-like organelles (Boreham and Stenzel, 1993, Dunn *et al.*, 1989, Tan *et al.*, 1974, Zierdt, 1973, Zierdt, 1978, Zierdt, 1991a, Zierdt *et al.*, 1967, Zierdt and Tan, 1976a, Zierdt and Tan, 1976b). The organism has a number of morphologically distinct forms (Boreham and Stenzel, 1993, Dunn *et al.*, 1989, Tan *et al.*, 1974, Tan and Zierdt, 1973, Zierdt, 1991a, Zierdt and Nagy, 1993, Zierdt *et al.*, 1967). Forms of *Blastocystis*, with the exception of cyst forms, are not bordered by a cell wall but are enclosed by a bilaminar membrane (Mehlhorn, 1988, Zaman *et al.*, 1995b, Zierdt, 1991a). Physiologically, *Blastocystis* spp. do not show any growth in fungal medium and growth is optimal at 37 °C and neutral pH. Growth is not inhibited by antifungal agents such as amphotericin B (Dunn and Boreham, 1991, Zierdt, 1973, Zierdt, 1986, Zierdt *et al.*, 1967, Zierdt and Williams, 1974, Zierdt *et al.*, 1995). These characteristics appear to link the organism more with protozoa than the fungi.

On the basis of suggested means of multiplication and its morphological and culture characteristics, *Blastocystis* was classified by Zierdt (1978) in the subphylum Sporozoa, and a new class Blastocystea and new order Blastocystida were proposed. Later, Zierdt (1988) classified this organism to the subphylum Sarcodina (amoebas), in a separate suborder, Blastocystina, but data to support this reclassification were not been provided.

Jiang and He (1993) later proposed a new subphylum Blastocysta which included the class Blastocystea, the order Blastocystida, the family Blastocystidae and genus *Blastocystis*, with

*B.hominis* as the type species. Although it was clear that *Blastocystis* did not fit well into the existing protozoa classification, there was not enough proof to raise a new classification for the organism (Boreham and Stenzel, 1993, Hollebeke and Mayberry, 1994).

As the effect of the physiological state on the ultrastructural appearance of the organism became clear (see section 1.3), it was obvious more morphological and molecular data were needed (Zierdt, 1993). The taxonomy and phylogenetic affinities of *Blastocystis* have been analysed by comparison of the parasite's small subunit (SSU) rRNA gene sequence with those from different eukaryotes. By analysis of partial SSU rRNA sequence it was shown that *Blastocystis* is not monophyletic with yeast (*Saccharomyces*) or sarcodines (Johnson *et al.*, 1989). Based on these data, Johnston *et al.* concluded that *Blastocystis* should be placed as an outgroup of the clade that links ciliates and apicomplexa.

In a subsequent study (Silberman *et al.*, 1996) the complete *Blastocystis* SSU rRNA gene was sequenced and it showed that *Blastocystis* should be placed within the stramenopiles. This diverse group includes slime nets, water moulds and brown algae. This study also indicated that *Blastocystis* was, surprisingly, phylogenetically related to the flagellate *Proteromonas* (Silberman *et al.*, 1996). Both these organisms share certain life-cycle traits. They are gut endosymbionts of vertebrates and encyst to an environmentally resistant form that appears to allow transmission between hosts. However, unlike *Proteromonas*, *Blastocystis* does not have flagella and tubular hairs. Fig 1.1 showed the close phylogenetic position of Stramenopiles with the Alveolates (which include ciliates and apicomplexa) and Rhizaria (which include foraminifera and other free-living protozoa) groups.

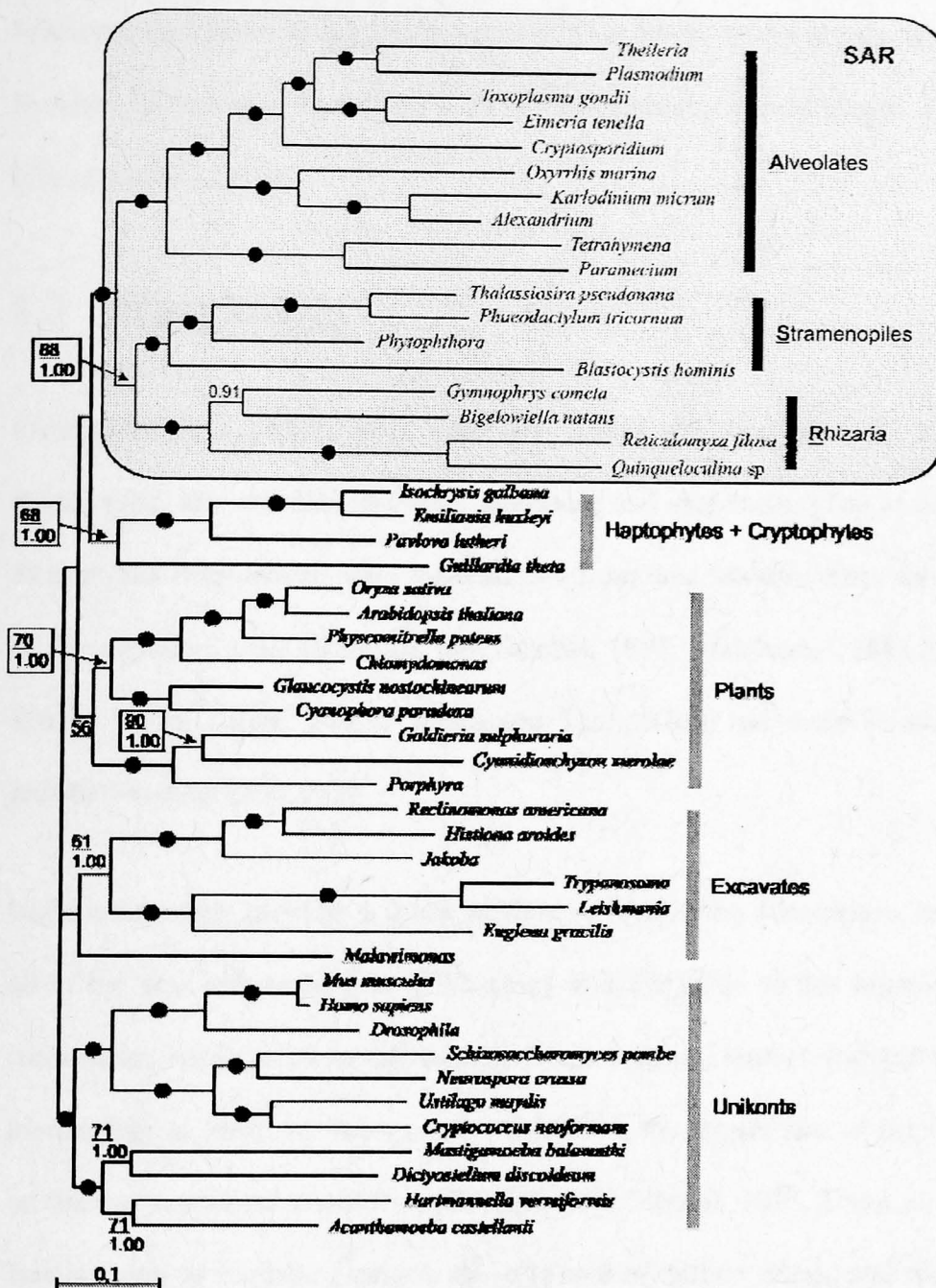


Figure 1.1. The position of *Blastocystis* within Stramenopiles. Black dots represent values of 100% bootstrap support (BP) and Bayesian posterior probabilities (BiPP) of 1.0. Nodes without numbers correspond to supports weaker than 50% BP and 0.8 BiPP. Both maximum likelihood and Bayesian analysis were used based on concatenated sequences of 126 genes. This figure is taken from Burki *et al.*, 2007

In the revised classification of the six-kingdom system of life (Cavalier-Smith, 1998) *Blastocystis* is placed in a newly created super-group Chromalveolata, within the subdivision Stramenopiles, and subgroup Opalinata (Adl *et al.*, 2005). *Blastocystis* is the first

stramenopile known to parasitise humans. One of the *Pythium* spp. which cause pythiosis, *Pythium insidiosum*, is the only other stramenopile which causes disease in mammals (Gaastra *et al.*, 2010).

## 1.3 Morphology

*Blastocystis* is a polymorphic organism. There are four generally recognised forms of *Blastocystis*, the vacuolar, granular, amoeboid and cyst forms (Tan *et al.*, 2002). Reports of *Blastocystis* from human stool material and from host intestine have shown that other forms of the organism exist (Boreham and Stenzel, 1993, Mehlhorn, 1988, Zaman *et al.*, 1995b, Zierdt, 1991a, Zierdt, 1991b, Zierdt and Tan, 1976b) but these forms have not yet been included in diagnostic texts.

Light microscopy provides a quick method of identifying *Blastocystis* in samples but nearly all of the new information on cell biology and life cycle of this organism has been gained from transmission electron microscopy. Ultrastructural studies showed that a continuum of morphological forms of *Blastocystis* exists, with the appearance of the organism depending on the environmental conditions (Boreham and Stenzel, 1993, Dunn *et al.*, 1989). Physical factors, such as osmotic changes, the presence of certain drugs, and metabolic status, may have an effect on the morphology of the organism both in vivo and in vitro (Stenzel and Boreham, 1996).

### 1.3.1 Vacuolar form

The vacuolar form, also called the vacuolated or central vacuole form, has been considered to be the typical *Blastocystis* cell form (Zierdt, 1991a). It is generally spherical, and therefore

shows a rounded shape in transmission electron micrographs, even though some with irregular shape are also present (Boreham and Stenzel, 1993). The vacuolar form is the main form in axenic and xenic liquid culture (Fig1.2) and is frequently seen in fresh faecal samples (Tan *et al.*, 2002). It can fluctuate broadly in size ranging from 2 to 200  $\mu\text{m}$  in diameter with average diameters of 4-15  $\mu\text{m}$  and there is a variability in the morphology of vacuolar contents (Stenzel and Boreham, 1996). Both nucleus and cytoplasm are compressed at the periphery of the cell by a large central vacuole. *Blastocystis* cells may contain up to four nuclei and these are spaced more or less evenly around the cell (Zierdt, 1973). This vacuolar form reproduces rapidly by binary fission (Zierdt *et al.*, 1967).

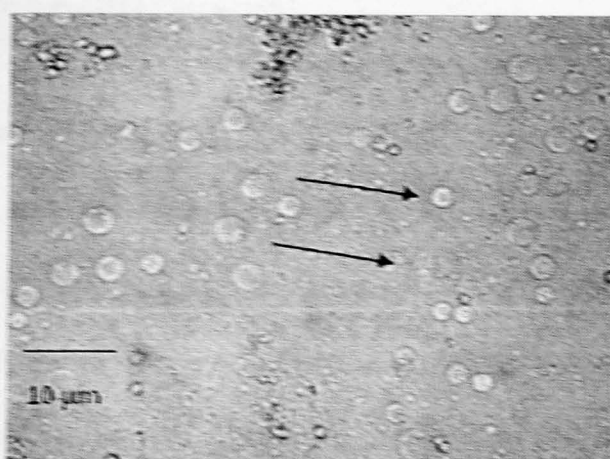


Figure 1.2. Vacuolar forms of *Blastocystis* in vitro in xenic culture showing extensive size variation (arrows).

Under electron microscopy, the vacuolar form contains a characteristic large vacuole and a thin rim of peripheral cytoplasm. As expected from light microscopy, organelles such as the nucleus, Golgi and mitochondria-like structures are located within the cytoplasmic rim (Tan *et al.*, 2001). The exact function of the central vacuole is presently unclear. It has been suggested to act as a storage organelle or to function in endodyogeny and schizogony, two proposed methods of reproduction, through the development of reproductive granules (Dunn *et al.*, 1989, Suresh *et al.*, 1994, Zierdt, 1988, Zierdt, 1991a) but these functions have not

been supported by further studies. Other distinct but less frequent forms been described, such as avacuolar and multivacuolar cells, and cells having filament-like inclusions (Stenzel and Boreham, 1996).

### **1.3.2 Granular form**

The granular form is commonly observed in old and non-axenized cultures (Tan, 2004). Granular forms usually are slightly larger than the average vacuolar forms, and diameters of 15 to 25  $\mu\text{m}$  (Zierdt, 1973), 3 to 38  $\mu\text{m}$  (Tan *et al.*, 1974), and 6.5 to 19.5  $\mu\text{m}$  (Dunn *et al.*, 1989) have been reported.

Apart from the contents of the central vacuole, the granular form is similar in appearance to the vacuolar form. Granular forms also display a thin peripheral band of cytoplasm surrounding a large central vacuole. There is a difference in the morphology of the vacuolar contents. In the vacuolar form, the central vacuole usually contains finely granular material which is scattered throughout the vacuole (Dunn *et al.*, 1989). In the granular form, the central vacuole may contain granules with many morphological types.

Two granular types were proposed based on light microscopy studies. It was suggested that one granule type developed into daughter *Blastocystis* cells while the other had a role in metabolism (Zierdt *et al.*, 1967). Later, with help of transmission electron microscopy, it was revealed that three types of granules, described as metabolic, lipid, and reproductive granules, were present in the cells (Tan and Zierdt, 1973).

It was suggested that the metabolic granules were cytoplasmic, the lipid granules were found in central vacuole and cytoplasm, and the reproductive granules were present in the central

vacuole (Zierdt, 1973). These observations have not been confirmed and the study by Dunn *et al.* (1989) described myelin-like inclusions, crystalline granules, small vesicles and lipid droplets in the central vacuole of granular forms.

Different conditions are known to induce granular form production from the vacuolar form, including (i) increased serum concentration in the culture medium (Dunn *et al.*, 1989, Zierdt, 1973), and (ii) transfer of cells to a different culture medium (Stenzel *et al.*, 1991) or axenization of the culture (Tan *et al.*, 1974). Vdovenko (2000) illustrated that the vacuolar form is an artifact of culture conditions and may not be part of the life cycle of this organism, which supports the view of Stenzel *et al.* (1991). Occasionally, the granular form has been observed in cultures without increased serum concentration (Dunn *et al.*, 1989).

Although a number of mechanisms for division of vacuolar and granular forms have been described (Boreham and Stenzel, 1993, Jiang and He, 1993, Zierdt, 1991a), the only confirmed mode of reproduction of these forms of *Blastocystis* is binary fission.

### **1.3.3 Amoeboid form**

The amoeboid form of *Blastocystis* is present in culture and stools in small numbers (Fig.1.3). It is smaller than both vacuolar and granular forms, the cell size ranging from 2.6  $\mu\text{m}$  to 7.9  $\mu\text{m}$ , and there are conflicting descriptions of its morphology, especially using electron microscopy (Dunn *et al.*, 1989, Stenzel and Boreham, 1996, Tan *et al.*, 2002).



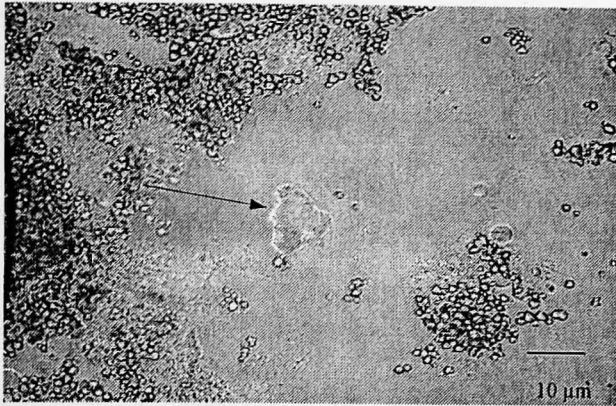


Figure 1.3. Amoeboid form of *Blastocystis* occasionally seen in culture showing pseudopod-like cytoplasmic extensions (arrow)

According to Zierdt (1973) this form appeared in small numbers in older cultures and those treated with antibiotics, and occasionally in faecal samples. The form has no central vacuole, the cells were reported to feed on bacteria, and one or two nuclei were seen at the centre of the cell.

In contrast, in another report the amoeboid cells were said to contain a large central vacuole, and cultures with high serum content showed electron-lucent granules were present in the central vacuole of the organisms. In most specimens a distinct, permanent cell wall or membrane is not seen (Tan and Zierdt, 1973). Engulfed bacteria were seen in lysosome-like bodies within the cell (Dunn *et al.*, 1989).

The way amoeboid cells multiply is unconfirmed. Some modes of division that have been suggested included budding (Zierdt, 1973) and plasmotomy (Zierdt, 1991a). However, there is no conclusive evidence to support either of these reproductive modes.

There has been a suggestion that the amoeboid form is an intermediate between the vacuolar and cyst forms of *Blastocystis*, but this has not been proven (Singh *et al.*, 1995, Suresh *et al.*,



1994). These reports also proposed that bacteria were ingested by the amoeboid form to supply nutrition for the encystation process.

### 1.3.4 Cyst form

Cyst-like forms of *Blastocystis* were first discovered in the fresh faeces of a patient with acquired immunodeficiency syndrome by Mehlhorn, (1988), but Stenzel and Boreham (1991) provided the first full morphological description of the cyst. The cyst forms were observed in faecal material, especially material that had been stored for many days before being fixed, and occasionally in laboratory cultures. The cyst appeared rounded or rarely ovoid by transmission electron microscopy (Stenzel and Boreham, 1991).

Zaman *et al.* (1995b) also have provided an ultrastructural description of the cyst form and have given a method for concentration of this form from faecal material by repeated washing in distilled water and centrifugation on Ficoll-Paque. The studies by Suresh (Suresh *et al.*, 1994, Suresh *et al.*, 1993) were said to identify the cyst form, but the micrographs in these reports show organisms morphologically identical to the granular form described by other people (Boreham and Stenzel, 1993, Dunn *et al.*, 1989, Tan and Zierdt, 1973).

The cyst forms of *Blastocystis* are smaller than vacuolar and granular forms found in cultures. Their size is reported to be 5 to 10  $\mu\text{m}$  (Mehlhorn, 1988), 3.7 to 5  $\mu\text{m}$  (Stenzel and Boreham, 1991), 3 to 6  $\mu\text{m}$  (Zaman *et al.*, 1995a) and 2 to 5  $\mu\text{m}$  (Moe *et al.*, 1996) in diameter. Multiple mitochondrion-like organelles have been distinguished in the cyst (Zaman *et al.*, 1995a) and up to four nuclei per cyst have been described by Mehlhorn (1988), although Stenzel and Boreham (1991) discovered only one nucleus per cyst form from human faeces.

*Blastocystis* cysts isolated from animal faecal material show distinct morphological differences when compared with faecal cysts from humans. Large multinucleated cyst forms of *Blastocystis* were discovered in faecal material from Macaque monkeys. These cyst forms measured up to approximately 15 µm in diameter. Multiple individual cysts surrounded by a single fibrillar layer were detected from chicken faecal material (Stenzel *et al.*, 1997) but other cyst forms with a bare cyst wall were also found. Individual cysts inside the multiple cyst form measured roughly 3-4 µm in diameter and were shown to be uninucleate. The authors proposed that the distinct morphologies of the cyst forms possibly reflected the presence of different *Blastocystis* species.

Viability investigation of *Blastocystis* cysts showed that they do not lyse in water (Zaman *et al.*, 1995a) and are capable of living at room temperature for up to 19 days (Suresh *et al.*, 1993) but are sensitive to excessive heat and cold and to common disinfectants (Moe *et al.*, 1996). In comparison to the cystic forms, the vacuolar and granular forms are susceptible to alteration in temperature and also hypertonic and hypotonic environments and exposure to air (Zierdt, 1991a, Boreham and Stenzel, 1993, Matsumoto *et al.*, 1987, Singh *et al.*, 1995, Zierdt, 1973) and hence appear unlikely to provide a mode of transmission. Thus, the only transmission form of the parasite is the cystic stage (Yoshikawa *et al.*, 2004c, Tan *et al.*, 2002). According to Yoshikawa *et al.* (2004c) the cyst form is considered as the only transmissible stage of this parasite.

## 1.4 Life cycle and transmission

The mode of transmission and life cycle of *Blastocystis* are little understood. Different life cycles have been suggested for *Blastocystis* by different authors (Boreham and Stenzel, 1993, Singh *et al.*, 1995, Zierdt, 1973). Arguments about the methods of division of the parasite

have existed from the beginning. The first to propose a life cycle for *Blastocystis* was Alexeieff in 1911 (cited in Boreham and Stenzel, 1993a). He illustrated a complex life cycle which involved binary fission of a binucleate stage (plasmotomic division), and autogamy, a sexual phenomenon, to produce primary cysts. The cysts created spores by multiple budding. These spores were considered to be resistant forms. Another mode of reproduction described by Alexeieff is merogony. The accuracy of this life cycle was questioned by a number of authors (Lynch, 1917; Cifferi and Redaelli, 1938; Lavier, 1952) (cited in Boreham and Stenzel, 1993a) even though binary fission (also known as plasmotomic division) appears to have been readily accepted and observed in a number of studies (Dunn *et al.*, 1989, Tan and Zierdt, 1973, Yamada *et al.*, 1987, Zierdt, 1991a, Zierdt *et al.*, 1988, Zierdt and Swan, 1981).

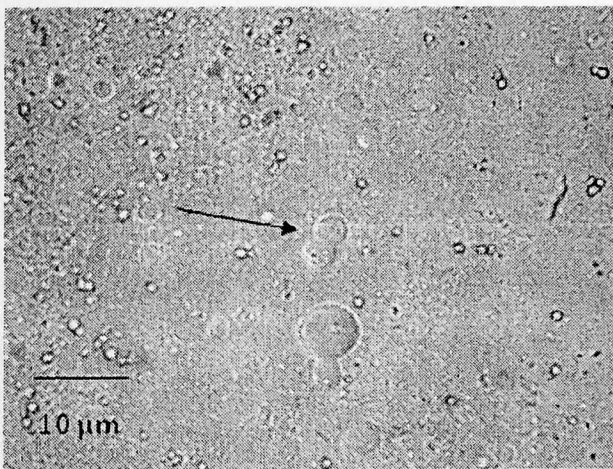


Figure 1.4 Cells of *Blastocystis* undergoing binary fission in culture (arrow).

A new life cycle for *Blastocystis* was proposed by Zierdt in 1973 based on his microscopy observations. It shows that the vacuolar form develops into either the granular form, which later releases vacuolar daughter cells within the central vacuole, or to the amoeboid form, which produces vacuolar daughter cells by budding division. Boreham and Stenzel (1993) have declared that there is no certainty on the role of the amoeboid form in the life cycle of *Blastocystis*. It is not even known whether the amoeboid form can divide by binary fission.

Uncertainty continued as a report by Zierdt *et al.* (1988) indicated that binary fission (Fig.1.4) and plasmotomy were two different forms of multiplication.

At least four modes of multiplication were described by Zierdt (1991a) for *Blastocystis* and all of them are asexual: binary fission, plasmotomy, endodyogeny and schizogony. However, the links between the modes of division were not demonstrated and little proof was presented for their existence. Sexual reproduction was not illustrated in *Blastocystis* in his paper.

Another life cycle was proposed by Singh *et al.* (1995); these authors propose a life cycle of *Blastocystis* involving both thin and thick-walled cysts. They suggested that the thick-wall cysts are responsible for outside transmission through the faecal-oral route, whereas the thin walled cysts are autoinfectious leading to multiplication of the organism within the intestine. Progeny arise by schizogony within the precyst form, resulting in a thick-walled cyst, which in turn ruptures to liberate daughter vacuolar forms. In Singh's paper another cycle of autoinfection was also postulated, although this is not consistent with the description of autoinfection associated with other parasites, like *Enterobius* and *Strongyloides* spp. In this cycle, the vacuolar form differentiates into the thin-walled cyst form through the multivacuolar and precyst forms. They propose that schizogony is occurring within the thin-walled cyst which later ruptures to release daughter vacuolar forms.

Boreham and Stenzel (1993) suggested a simple life cycle of *Blastocystis*. The main form in the colon of humans is a small non-vacuolated cell without a surface coat. As this form passes through the colon, the small vesicles present in the cytoplasm coalesce to produce the multivacuolar stage found in faeces, which is encircled by a thick surface coat. The cyst wall seems to form underneath the surface coat which afterwards appears to slough off. The resulting cyst form is likely to be the infective stage of *Blastocystis*. Initial support for this

theory has been supplied by a study showing that the cyst form is infective to rats (Suresh *et al.*, 1993). Little information exists on the origin of amoeboid forms since only a small number of amoeboid cells have been found in any samples. It is likely that the amoeboid form develops from the avacuolar form since there are morphological resemblances, as described by Dunn *et al.* (1989). Boreham and Stenzel (1993) proposed that the other forms are culture artefacts and not part of the life cycle in vivo.

Additional experimental confirmation of this process of cyst formation and the ability of cyst stages to survive in external environmental conditions is essential. Infection of another host and excystation of the cell would complete the cycle. Since the cyst is more common in stored faecal material than in fresh stools, Stenzel and Boreham (1993) suggest that this form may develop in response to excretion from the host or to external environmental factors. Very little information is available.

The mode of transmission of *Blastocystis* remains in question; although it is assumed that transmission may take place through the faecal-oral route, few studies have examined this point (Kain *et al.*, 1987). Transmission of *Blastocystis* can be assisted by the contamination of environment, food, or water with excreted cysts from the hosts (Leelayoova *et al.*, 2008). Waterborne transmission of *Blastocystis* by the use of unboiled, untreated water or poor sanitary conditions has been reported (Leelayoova *et al.*, 2008, Nimri and Batchoun, 1994, Nimri, 1993). Doyle *et al.* (1990) suggested that the close association of humans and animals could be a source of transmission of *Blastocystis*. Also Rajah Salim *et al.* (1999) have found that people who come into close contact with animals have more chance of having a *Blastocystis* infection. In contrast, another study by Nimri and Batchoun (1994) did not find any relationship between *Blastocystis* and presence of animals. Transmission of *Blastocystis*

has been reported among family members in Italy (Guglielmetti *et al.*, 1989), suggesting that other animals are not always involved.

In reviewing the literature on *Blastocystis* none of the publications report anything about ploidy of *Blastocystis* during its life cycle and there is no convincing evidence for sexual reproduction (Tan *et al.*, 2008).

In summary, the *Blastocystis* mode of transmission is through the faecal-oral route, binary fission is the only accepted mode of cell division, and the cyst is the infective stage.

## 1.5 Pathogenicity

The most difficult question concerning *Blastocystis* today is whether this organism is truly pathogenic or a commensal, or is pathogenic only under specific conditions such as immunosuppression, poor nutrition or concurrent infections. Attempts to link pathogenicity to *Blastocystis* by epidemiological studies have been criticized due to the difficulty to exclude all other infectious and non-infectious causes, especially when it is considered that 25% of patients with diarrhea have no known etiology (Edmeades *et al.*, 1978 cited in Boreham and Stenzel (1993). Although many authors have suggested that *Blastocystis* causes disease there are also a numbers of reports to contrary (Table 1.1)

Table 1.1 Different survey and epidemiology studies suggesting pathogenic and non-pathogenic *Blastocystis*

Reference	Pathogenic	Nonpathogenic	Sample groups	Size of the study		Type of technique	Type of study	Country
				No of patient	No of samples			
Garcia et al., 1984	✓		Patients	289/196 <sup>⊙</sup>	485	Formalin/ethyl acetate concentration and trichrome permanent stained smear	Retrospective chart review	Los Angeles, USA
Babcock et al., 1985	✓		Out Patients	400/3600 <sup>⊙</sup>	4000	Formalin/ethyl acetate concentration	Report study	Kathmandu, Nepal
Sheehan et al., 1986	✓		Patients	62/327 <sup>⊙</sup>	389	Unstained wet mount smear	Report study	New York, USA
Babb and Wagener, 1989	✓		Patients	76/2624 <sup>⊙</sup>	2700	Trichrome staining technique	Report study	California, USA
Qadri et al., 1989	✓		Patients	647/2242 <sup>⊙</sup>	2889	Formalin/ethyl acetate concentration and saline and iodine-stained wet mount smear	Clinical report	Riyadh, Saudi Arabia
Doyle et al., 1990	✓		Out Patients	124/19 <sup>⊙</sup>	143	Formalin/ethyl acetate concentration/ trichrome stain and the modified acid-fast stain	Prospective study	Vancouver, Canada
Zaki et al., 1991	✓		Patients (Children)	19/20 <sup>1</sup>	39	Formalin/ethyl acetate concentration and then wet faecal smear	Clinical report	Kuwait, Kuwait
Telabasic et al., 1991	✓		Patients	82/12 <sup>⊙</sup>	94	Unstained wet mount	Report study	Sarajewo, Bosnia
Waghorn and Hancock, 1991	✓		Patients	51/15 <sup>⊙</sup>	65	Formalin/ethyl acetate concentration and direct faecal smear using saline and 5% aqueous nigrosin (counterstain)	Prevalence report	High Wycombe, UK
Nimri et al., 1994	✓		School children	175/28 <sup>+</sup>	203	Wet mount preparation and formal-ether concentration	Prevalence of intestinal parasites	Irbid, Jordan
Jelinek et al., 1997	✓		Tourists	69/21 <sup>1</sup>	90	Direct microscopy using iron-haematoxylin-stain and sodium acetate-acetic acid-formalin /ethylacetate-concentration technique	Case control study	Munich, Germany
				400/305 <sup>□</sup>	705			
Tasova et al., 2000	✓		Patients	206/200 <sup>■</sup>	406	Wet mount smear using saline and iodine /Formalin/ethyl acetate concentration and trichrome-stained smear	Non-randomized study	Adana, Turkey
Kaya et al., 2007	✓		Patients	46/6 <sup>⊙</sup>	52	Wet mount smear using saline and iodine /Formalin/ethyl acetate concentration method and trichrome and Kinyoun acid-fast stain	Report study	Isparta, Turkey
Vogelberg et al., 2010	✓		Tourists	1 <sup>⊙</sup>	1	Direct wet faecal smear	Case report	Dresden, Germany
Chen et al., 2003		✓	Healthy adults	99/193 <sup>⊙</sup>	292	Merthiolate-iodine formalin method	Case control study	Taipei, Taiwan
Senay and MacPherson, 1990		✓	Consecutive patients	90/30 <sup>⊙</sup>	120	Formalin/ethyl acetate concentration, wet mount smear and iron-haematoxylin stain smear	Prospective study	Hamilton, Canada
Zuckerman et al., 1994		✓	Patients	18/32 <sup>*</sup>	50	Formalin/ethyl acetate concentration and iodine wet mount	Prospective study	Texas, USA
Markell and Udkow, 1993		✓	Patients	16/21 <sup>⊙</sup>	37	Trichrome-stained technique	Comparison between symptomatic & asymptomatic	Oakland, USA
				109/161 <sup>□</sup>	270			
Shlima et al., 1995		✓	Random patients	189/112 <sup>1</sup>	301	Direct wet faecal smear and Formalin/ethyl acetate concentration	Prospective study control study	Bangkok, Thailand
Church et al., 1992		✓	Gay men with HIV and without	49/9 <sup>*</sup>	58	Haematoxylin and eosin, periodic acid Schiff-Alcian blue, acid-fast, Warthin-Starry, trichrome and Giemsa stains	Pilot association study	Alberta, Canada
Miller and Minshew, 1988		✓	Consecutive patients	11 <sup>⊙</sup>	11	Direct wet faecal smear and trichrome-stained smear	Association of <i>Blastocystis</i> with clinical disease	Washington, USA
Honki et al., 1997		✓	Random patients	30/6392 <sup>⊙</sup>	6422	Direct wet faecal smear	Epidemiologic survey	Tokyo, Japan
<p> <sup>⊙</sup> With &amp; without <i>Blastocystis</i>  <sup>⊙</sup> <i>Blastocystis</i> with &amp; without symptoms  <sup>1</sup> <i>Blastocystis</i> with &amp; without diarrhoea  <sup>+</sup> <i>Blastocystis</i> alone &amp; with other pathogenic parasites  <sup>□</sup> No <i>Blastocystis</i> with or without diarrhoea  <sup>■</sup> Patients with &amp; without hematological malignancy  <sup>⊙</sup> <i>Blastocystis</i> patient with urticaria  <sup>*</sup> Patients with <i>Blastocystis</i> &amp; healthy control  <sup>□</sup> No <i>Blastocystis</i> with or without symptoms  <sup>*</sup> Gay men with &amp; without HIV  <sup>⊙</sup> Patients with symptoms of enteritis and having <i>Blastocystis</i> </p>								

Symptoms commonly linked to infection with *Blastocystis* are non-specific and include diarrhoea, abdominal pain, cramps or discomfort, and nausea (El-Shazly *et al.*, 2005, Graczyk *et al.*, 2005, Kaya *et al.*, 2007, Moghaddam *et al.*, 2005, Stensvold *et al.*, 2008, Gupta and Parsi, 2006, Motazedian *et al.*, 2008, Nigro *et al.*, 2003, Tasova *et al.*, 2000). Profuse watery diarrhoea has been recorded in acute cases (Logar *et al.*, 1994, Tan and Zierdt, 1973, Zaki *et al.*, 1991). Other signs and symptoms sometimes reported include faecal leucocytes (Diaczok and Rival, 1987, Kaya *et al.*, 2007), rectal bleeding (al-Tawil *et al.*, 1994, Bratt and Tikasingh, 1990) and eosinophilia (Katsarou-Katsari *et al.*, 2008). Fever has been reported especially in acute cases (Gallagher and Venglarcik, 1985, Stensvold *et al.*, 2008, Zaki *et al.*, 1991).

Several studies have been carried out to evaluate the epidemiology of *Blastocystis* by detecting the presence or absence of the organism and linking this to pathogenicity by dividing patients into symptomatic and asymptomatic groups. The analysis of the results from such studies is not straight forward as case definition varies, and the duration of *Blastocystis* carriage is not known nor is the incubation period. Therefore, if *Blastocystis* infections cause symptoms, we need to know whether these symptoms are acute, chronic or intermittent. Also in the evaluation of all studies in table 1.1, there was no consistent technique used to detect *Blastocystis*. Some stages may not be detected by direct microscopy or concentration techniques, and although culture is thought to be more sensitive this method is not always used.

Some people have used individual case study results to make a judgment about the pathogenicity of *Blastocystis*. For example, Vogelberg *et al.* (2010) reported the possible link of a *Blastocystis* infection with the simultaneous occurrence of gastrointestinal illness.



Resolution of symptoms coincided with elimination of the parasite, suggesting it might be the cause of the problem. However, disappearance of symptoms may be due to elimination of unidentified pathogens rather than to the treatment of *Blastocystis* (Moghaddam et al., 2005).

Miller and Minshew (1988) assessed a small number of hospitalized patients (11 patients). Most of these patients had other diseases that could have explained the gastrointestinal tract problems or had recently received antibiotics or anti-protozoal drugs. The authors reached the conclusion that *Blastocystis* was not a pathogen. Chen *et al.* (2003) found that most of the individuals harbouring *Blastocystis* were asymptomatic, and that the occurrence of gastrointestinal symptoms was similar whether individuals were positive or negative for *Blastocystis*. Others (Markell and Udkow, 1993; Miller and Minshew, 1988; Shlima *et al.*, 1995) also concluded that *Blastocystis* is usually not a pathogen.

Both endoscopy and biopsy results showed that *Blastocystis* does not invade the colonic mucosa in human patients (Diaczok and Rival, 1987, Doyle et al., 1990, Garavelli et al., 1991, Stenzel and Boreham, 1996). Edema and inflammation of the intestinal mucosa has been reported to be present (Garavelli et al., 1991, Kain et al., 1987, Lee et al., 1990, Russo et al., 1988), but one case report (al-Tawil *et al.*, 1994) described colonic ulceration detected by colonoscopy and biopsy. *Blastocystis* was found in superficial ulcers and both inflammatory cells and eosinophils were present in the cecum, transverse colon, and rectum of the patient. Endoscopy of the upper gastrointestinal tract did not show any abnormal epithelia. This has excluded the possibility of other known bacteria, viral and pathogenic parasites as well as *Clostridium difficile* toxin being the cause. Non-infectious causes of the symptoms were not found, and the only parasite found in biopsy material linked with inflamed areas of the gastrointestinal tract was *Blastocystis* (al-Tawil *et al.*, 1994).

In contrast, another study (Chen *et al.*, 2003) using upper gastrointestinal endoscopy and sigmoidoscopy showed that presence of the organism had no relationship with any intestinal lesions. Similarly, in a group of patients with *Blastocystis* infection, Zuckerman *et al.* (1994) did not find any evidence of significant intestinal inflammation by endoscopy. Intestinal permeability was assessed by excretion of a radioactive marker ( $^{51}\text{Cr}$ -EDTA) and no increase was found. Based on this they concluded that *Blastocystis* did not cause significant intestinal injury.

The first report of a possible relationship between intestinal obstruction and concomitant *Blastocystis* infection was provided by Horiki *et al.* (1999). Intestinal blockage and accumulation of stool as well as haemorrhage from cancerous lesions may lead to more growth of *Blastocystis*. According to Tasova *et al.* (2000) *Blastocystis* is a common parasite in patients with haematological malignancy (HM) and it should be taken into account as a potential pathogen in patients with gastrointestinal symptoms and HM who are being treated with metronidazole.

There seems to be no evidence for the role of protective immunity to *Blastocystis* at the community level (Ashford and Atkinson, 1992). Ashford and Atkinson claimed that if the organism is pathogenic, only a small percentage of the community might be immune so that any individual immunity will not interfere with the overall community pattern. Other authors suggest that there is protective immunity in *Blastocystis* due to the self-limiting nature of the infection and spontaneous elimination of the parasite (Nimri, 1993).

Most investigators agree that adults are more likely to be infected than children (Horiki *et al.*, 1997). However, several studies reported that older children and adults have a lower infection rate and fewer symptoms than younger children (Nimri and Batchoun 1994); this may reflect

the stimulation of immunity by previous infection. However, this point remains controversial, as other studies have detected higher rates of infection in adults than in children (Ashford and Atkinson, 1992, Doyle *et al.*, 1990, Logar *et al.*, 1994, Martin-Sanchez *et al.*, 1992, Qadri *et al.*, 1989, Sanad *et al.*, 1991, Suresh *et al.*, 2001).

Protozoa can be significant enteric pathogens in patients with human immunodeficiency virus (HIV) infection. A wide variety of bacterial, viral, and parasitic pathogens can cause severe diarrhoea in patients with low CD4+ cell counts. Disagreement exists as to whether *Blastocystis* should also be included among the infectious agents capable of causing HIV-related diarrhea.

The prevalence and clinical significance of *Blastocystis* were determined by Albrecht *et al.* (1995) in a cohort study of 262 patients with HIV infection carried out at a university hospital in northern Germany. *Blastocystis* was detected in faecal samples of 99 patients (38%) and detection rate was different between groups. Patients with acquired immunodeficiency syndrome (AIDS) were more likely to carry *Blastocystis* (46%) compared to patients in earlier stages of HIV infection (32%),  $P = 0.03$ . No association with clinical symptoms was confirmed and the presence of *Blastocystis* was frequently associated with concurrent isolation of other enteric pathogens and non-pathogens. Another study by Hailemariam *et al.* (2004) showed no significant difference between HIV/AIDS patients and a HIV seronegative control group in the presence of common intestinal parasites. However *Blastocystis* was more prevalent in HIV/AIDS patients than in controls ( $P < 0.05$ ). A recent study in Jakarta on 318 HIV patients reported parasitic intestinal infections in 84.3% of subjects with *Blastocystis* as the most frequent parasite found (72.4%) (Kurniawan *et al.*, 2009).

In a survey carried out in Dakar, Senegal by Gassama *et al.* (2001) among 594 patients from May 1997 to May 1999, 158 were HIV+ with diarrhoea (D+), 121 were HIV- D+, 160 were HIV+ without diarrhoea (D-), and 155 were HIV- D-. The main causes of diarrhoea were different according to HIV serostatus of patients and *Blastocystis* was considered to be an opportunistic pathogen, since it was identified only in HIV infected patients, with higher prevalence in adults with diarrhoea (2.5% in HIV+D+ patients; 0.6% in HIV+D- patients)

A study by Cegielski *et al.* (1993) investigated whether particular intestinal parasites are linked to HIV infection in Tanzanian children with chronic diarrhoea. All children were aged 15 months to 5 years and admitted to hospital with chronic diarrhoea, and were compared to age-matched controls. Three groups were compared: HIV-infected and non-HIV-infected children with chronic diarrhoea and HIV negative controls without diarrhoea. Although intestinal parasites were detected in approximately 50% of children in all 3 groups, 40% of those with chronic diarrhea were HIV-seropositive and *Blastocystis* was more common in HIV-infected children ( $p=0.05$ )

In contrast, Church *et al.* (1992) and Viriyavejakul *et al.* (2009) did not find any link between the presence of *Blastocystis* and diarrhoea symptoms in HIV infected patients.

*Blastocystis* can be divided into different subtypes (STs; see section 1.11) and a number of authors have proposed that specific subtypes might be linked to pathogenicity. Hameed *et al.* (2011) found *Blastocystis* ST3 in 61% of urticarial patients and concluded it was a strong indicative pathogen for urticaria. The amoeboid form was detected in symptomatic patients with urticaria suggesting that this form was linked to pathogenicity. In addition, Katsarou-Katsari *et al.* (2008) presented the case of a patient with acute urticarial lesions and minor gastrointestinal symptoms who had *Blastocystis* ST3 that produced amoeboid forms

throughout cultivation. Meanwhile, Stensvold *et al.* (2010) reported a case of *Blastocystis* ST2 infection probably connected with repeated gastrointestinal and urticarial symptoms. In contrast, Dogruman-Al *et al.* (2008) suggested that *Blastocystis* ST2 is non-pathogenic.

Some authors reached the conclusion that *Blastocystis* is pathogenic, because treatment led to a reduction of symptoms and elimination of parasite in patients with acute diarrhoea, including some who had been treated with prednisone for rheumatoid arthritis (Lee *et al.*, 1990) and in others taking only the drug against the parasite (Ertug *et al.*, 2009, Kaya *et al.*, 2007, Moghaddam *et al.*, 2005, Rossignol *et al.*, 2005).

Several publications and diagnostic texts suggest that the abundance of *Blastocystis* in stool samples is related to signs and symptoms of the infection and that the numbers of organisms seen in faecal smears should be recorded in pathological reports (Lee, 1991, Pikula, 1987, Sheehan *et al.*, 1986, Zafar, 1988, Zierdt, 1991b, Zierdt, 1993). Some investigators, like Garcia *et al.* (1984), Sheehan *et al.* (1986), and Kay *et al.* (2007), recognize *Blastocystis* as a pathogen when no other cause of intestinal disease is identified, especially if the parasite is found in large numbers on the faecal smear. Senay and MacPherson (1990) found that symptoms due to the parasite were not related to the quantity of the *Blastocystis* present in the stool sample. In contrast, Doyle *et al.* (1990) and Sheehan *et al.* (1986) proposed that *Blastocystis* is more likely to cause symptoms when present in large numbers, defined as > 5 organisms per oil field.

Other papers have found no correlation between the number of *Blastocystis* in stool samples and the presence or intensity of symptoms (Doyle *et al.*, 1990, Horiki *et al.*, 1999). A decrease in the number of *Blastocystis* or eradication of the organisms has been significantly correlated with clinical recovery (Kain and Noble, 1989) but Miller and Minshew (1988)

suggested that there is no correlation between resolution of symptoms and either antiprotozoal treatment or disappearance of *Blastocystis* from faecal samples. Until there is confirmation that *Blastocystis* can be a pathogen and that the number of organisms is connected to virulence, numbers of *Blastocystis* cells should still be recorded and not interpreted as insignificant. (Church *et al.*, 1992, Doyle *et al.*, 1990, Logar *et al.*, 1994, Rolston *et al.*, 1989, Zaki *et al.*, 1991).

*Blastocystis* is a group of genetically diverse organisms, some of which may cause chronic or acute infection in some immunocompetent humans. The way *Blastocystis* behave in humans is similar to *Giardia* and *Entameba histolytica*-expression of symptoms depends on parasite genotype, host genotype, host immunity, and age. Parasite genotype may vary geographically along with other factors. Despite increasing reports that *Blastocystis* has pathogenic potential, the true nature of its virulence is currently unconfirmed due to the limited numbers of studies.

In conclusion, considering that there are no reports clearly proving that *Blastocystis* is nonpathogenic and since there are several studies concluding the opposite, it should be considered as a possible pathogen when all other possible infectious or noninfectious causes of the symptoms present have been excluded.

## **1.6 *Blastocystis* and Irritable bowel syndrome**

Irritable bowel syndrome (IBS) is “a functional disorder of the gastrointestinal tract characterized by regular occurrence of abdominal pain or discomfort along with alteration in frequency or consistency of the stool in the absence of organic etiology” (Agrawal *et al.*, 2008, Sorouri *et al.*, 2010, Yale *et al.*, 2008). It is a very common disorder with a worldwide prevalence of 10-20% (Longstreth *et al.*, 2006).

Young adult patients are more frequently diagnosed with IBS than people over the age of 50 years (Drossman *et al.*, 2002) and most studies find a female predominance (Nam *et al.*, 2010, Park *et al.*, 2010) although only a few people see their family doctor. The disease results in a reduced quality of life and is a multi-billion pound health-care problem (Talley and Spiller, 2002). In the UK, only 50% of IBS cases are thought to be diagnosed (Spiller *et al.*, 2007).

The symptoms of IBS differ from one person to another and may include irregular bowel movements, abdominal pain or discomfort, flatulence, and diarrhoea or constipation (Salonen *et al.*, 2010). Stress worsens IBS rather than being causative in any way (Agrawal *et al.*, 2008).

According to the widely used Roma II criteria (Table 1.2) IBS sufferers can be clustered into three symptom groups based on the stool appearance, stool frequency and defecatory symptoms: diarrhoea predominant (IBS-D), constipation predominant (IBS-C), and mixed type (IBS-M) with alternating occurrence of both diarrhoea and constipation (Longstreth *et al.*, 2006, Sorouri *et al.*, 2010, Khan and Chang, 2010).

Table 1.2 Roma II Criteria (Li *et al.*, 2003)

At least 12 weeks or more, which need not be consecutive, in the previous 12 months of abdominal pain or discomfort that has 2 of 3 features:
Relief with defecation
Onset associated with a change in the frequency of stool
Onset associated with a change in the form (appearance) of stool

More recently, the Roma III criteria (Table 1.3), which concentrate on the symptom frequency over persistence, have been issued (Longstreth *et al.*, 2006).

Table 1.3 Roma III Criteria (Dong *et al.*, 2010)

Recurrent abdominal pain or discomfort at least 3 days per month in the last 3 months associated with 2 or more of the following
Improvement with defecation
Onset associated with a change in frequency of stool
Onset associated with a change in form (appearance) of stool

The cause and pathophysiology of IBS are complicated and not well explained, and the main significant abnormalities include visceral hypersensitivity, irregular gut motility and autonomous nervous system dysfunction (Salonen *et al.*, 2010). Barbara *et al.* (2009) demonstrated that there is reliable evidence showing that IBS may be the adverse result of an acute episode of infectious gastroenteritis, the so-called postinfectious (PI) IBS, and the infectious agents involved in the development of IBS include pathogenic bacteria, parasites, and viruses. Studies have illustrated that genetic factors, chronic stress and enteric infections can predispose persons to developing IBS (Khan and Chang, 2010). Most drug therapies to date are unable to make a major impact on the quality of life for sufferers (Khan and Chang, 2010). Hence, an important problem is to understand what lies behind the development of symptoms in IBS (Thompson, 2005) .

An understanding of the role of *Blastocystis* in IBS is restricted by the ambiguity surrounding its pathogenicity (Stark *et al.*, 2007). Nevertheless, symptoms that have been attributed to infection with *Blastocystis* are non-specific, IBS-like and include diarrhoea, abdominal pain,



cramps or discomfort, and nausea (Doyle *et al.*, 1990, Zaki *et al.*, 1991). Furthermore, chronic excretion of *Blastocystis* with persistent symptoms has been reported (Zaki *et al.*, 1991).

Hussain *et al.* (1997) showed that IgG antibody levels to *Blastocystis* in patients with IBS were significantly higher compared with asymptomatic controls, demonstrating immune activation, and suggesting some association between *Blastocystis* and IBS. In a study by Yakoob *et al.* (2004) *Blastocystis* was more frequently demonstrated in the faecal samples of IBS patients (46 %) than the control group (7%). Giacometti *et al.* (1999) evaluated a possible link between *Blastocystis* infection and IBS and their findings support a link between the two. In contrast, Tungtrongch *et al.* (2004) found no relationship between presence of *Blastocystis* in faeces and IBS diagnosis. In chapter 4 this study will be discuss in more detail.

Some authors have suggested that an intestinal tract that is abnormal for any reason may provide conditions suitable for proliferation of *Blastocystis* (Udkow and Markell, 1993, Neal *et al.*, 1997). It is possible that *Blastocystis* infection is an indicator of intestinal dysfunction or resident intestinal flora disorders rather than a cause of IBS. Whenever *Blastocystis* is detected in stool samples of patients with IBS it does not necessary mean that the symptoms are due to this organism and other infective and non-infectious causes should be investigated (Stark *et al.*, 2007).

In summary, accumulating reports suggest an association between *Blastocystis* infection and IBS. However, it is unclear whether *Blastocystis* is a primary etiological agent in IBS, and it has been suggested that an abnormal intestinal situation like IBS may give an environment in which parasite numbers can increase (Udkow and Markell, 1993).

## 1.7 Treatment of *Blastocystis*

The necessity for treatment of *Blastocystis* infections remains controversial (Stenzel and Boreham, 1996). In the absence of certain proof of pathogenicity of the organism, treatment with possibly unsafe drugs and the failure to examine the true cause of symptoms in patients are of concern to many physicians (Markell and Udkow, 1986).

Most data on the treatment of *Blastocystis* are case control studies to investigate and evaluate both the clinical findings and the effect of drugs like metronidazole, trimethoprim-sulfamethoxazole (TMP-SMX), nitazoxanide and paromomycin in patients with *Blastocystis*. The main problem in evaluation of these studies is the diagnostic methods used to detect the organism. Stool microscopy is the most common technique and culture was used in one study only, by Stensvold *et al.* (2008).

There are a number of papers discussing the treatment of *Blastocystis* but only two papers described a controlled treatment trial; these studies were carried out in Egypt and Italy.

One of the control treatment trials described by Nigro *et al.* (2003) between April 1999 and October 2000, consisted of 616 subjects with diarrhoea referred to an outpatient clinic of the University of Catania. Five hundred were referred by a primary care physician; 116 arrived from other sections of the unit. Three fresh stool specimens were collected from each of the 616 subjects. All stool specimens were examined by direct wet mounted smear, trichrome staining, flotation and formol-ether concentration techniques.

A total of 76 patients had *Blastocystis* infection as the only potential cause of diarrhoea. Of the 76 enrolled subjects, 40 were randomly assigned to group A (treatment), and 36 to group B (placebo).

Table 1.4 Faecal results and Symptoms of Metronidazole-treated (n=40) and Placebo-treated (n=36)

Subjects at 1 and 6 Months post-treatment				
	1 Month post-treatment		6 Months Post-treatment	
	Metronidazole	Placebo	Metronidazole	Placebo
<i>Blastocystis</i> positive				
Symptomatic	5(13%)	31(86%)	10(25%)	24(67%)
Asymptomatic	3(7%)	4(11%)	11(28%)	7(19%)
<i>Blastocystis</i> negative				
Asymptomatic	32(80%)	1(3%)	19(48%)	5(14%)

Metronidazole treatment was a single 1.5-gram dose (as six 250-mg pills) orally per day for 10 consecutive days. This trial resulted in the resolution of symptoms in 87.5 % of treated subjects one month after treatment compared to 14% in the placebo group. *Blastocystis* was eradicated from the stool in 80% of individuals who received metronidazole vs. only 3% in the placebo group. Seventy five percent of subjects in the treatment group were asymptomatic at the end of 6 months compared to 33% in placebo group (Nigro *et al.*, 2003). This study compared the efficiency of metronidazole with a placebo in the treatment of diarrhoea in which *Blastocystis* was the only organism found. Metronidazole was observed both to reduce diarrhoea and achieve a higher rate of parasitic clearance. The extension of follow-up to 6 months resulted in an increase of parasitic relapses in the metronidazole-treated group both symptomatic and asymptomatic. It is not known whether the recurrence of *Blastocystis* is related to recrudescence or to reinfection.

A second clinical trial evaluated the efficiency of Nitazoxanide for the treatment of diarrhoea and enteritis associated with *Blastocystis*. This is a Randomised, double-blind, placebo-controlled study was carried-out in Egypt (Rossignol *et al.*, 2005)

Different formulae of Nitazoxanide were used in this study, including 500mg orally for patients 12 years or older and 200 mg or 100mg twice daily for three days orally for children between 4-11 and 1-3 years, respectively.

Four days after completion of therapy, 36 (86%) of the 42 patients who received Nitazoxanide showed resolution of symptoms compared with 16 (38%) of 42 patients who received a placebo ( $P < 0.0001$ ). Thirty-six (86%) of 42 patients receiving Nitazoxanide were also clear of *Blastocystis* organisms in the post treatment stool samples compared with only 5 (12%) of 42 patients who received the placebo ( $< 0.0001$ ). There was no difference in response rate between patients who received the drug as tablets or as a suspension.

These studies suggest that *Blastocystis* is pathogenic in some patients and can be treated successfully with nitazoxanide or metronidazole. It is unclear whether the effect on *Blastocystis* is direct or indirect through its effect on the bacterial flora. The high rate of recurrence after 6 months in the study by Nigro *et al.* (2003) suggests that *Blastocystis* may be suppressed rather than eliminated by metronidazole treatment, although reinfection cannot be ruled out.

## 1.8 Epidemiology and prevalence

The epidemiology of *Blastocystis* remains incompletely known. *Blastocystis* is often the most frequent parasite reported in human faecal samples, both from symptomatic patients (Pikula, 1987, Vickerman, 1994, Zierdt *et al.*, 1995) and from healthy individuals (Ashford and Atkinson, 1992).

**Table 1.5** Prevalence of *Blastocystis* infections in different countries

Prevalence (%)	Country	Reference
0.5	Japan	(Horiki <i>et al.</i> , 1997)
2.1	Turkey	(Koksal <i>et al.</i> , 2010)
3.2	Canada	(Doyle <i>et al.</i> , 1990)
3.7	China	(Wang <i>et al.</i> , 2002)
7	Mexico	(Diaz <i>et al.</i> , 2003)
8	Canada	(Senay and MacPherson, 1990)
11.3	Germany	(Jelinek <i>et al.</i> , 1997)
13.5	Thailand	(Yaicharoen <i>et al.</i> , 2006)
13.6	Italy	(Cirioni <i>et al.</i> , 1999)
14.1	Taiwan	(Cheng <i>et al.</i> , 2003)
14.2	Turkey	(Ostan <i>et al.</i> , 2007)
14.7	India	(Rayan <i>et al.</i> , 2010)
14.9	Malaysia	(Suresh <i>et al.</i> , 2001)
15	Germany	(Paschke <i>et al.</i> , 2010)
16.3	Egypt	(Sanad <i>et al.</i> , 1991)
20	USA	(Amin, 2002)
22.9	Argentina	(Minvielle <i>et al.</i> , 2004)
25	Jordan	(Nimri, 1993)
26.5	Brazil	(Nascimento and Moitinho Mda, 2005)
26.6	Libya	(Alfellani <i>et al.</i> , 2007)
30	Thailand	(Shlim <i>et al.</i> , 1995)
33.3	Egypt	(Rayan <i>et al.</i> , 2007)
36.4	Colombia	(Londono <i>et al.</i> , 2009)
40.7	Philippines	(Baldo <i>et al.</i> , 2004)
40.9	Brazil	(Aguiar <i>et al.</i> , 2007)
52.3	Malaysia	(Noor Azian <i>et al.</i> , 2007)
61.8	Chile	(Torres <i>et al.</i> , 1992)

The incidence of infection with *Blastocystis* in a range of communities has been investigated (Table 1.5) but the results must be treated with caution since most studies were based on samples submitted to parasitology laboratories and therefore are mostly related with symptomatic patients (Boreham and Stenzel, 1993) and the methods used vary. Due to the variation of *Blastocystis* prevalence between studies within the same country I have compared different studies in Turkey and Malaysia to see why these differences may occur.

Table 1.6 compares two studies carried out by different authors in different areas of Turkey. Koksal *et al.* (2010) collected faecal samples from an outpatient group in Istanbul whereas

Ostan *et al.* (2007) obtained faecal samples from random students at a primary school in Manisa.

**Table 1.6** Comparison of *Blastocystis* prevalence in two studies in Turkey

Authors	Koksal <i>et al.</i> (2010)	Ostan <i>et al.</i> (2007)
Type of patient	Out patients suspected of having intestinal parasites	Children in primary school
Age group	19-40 years (young adult)	9-12 (children)
Location	Istanbul city	Manisa city
No of sample stool samples	27664	294
Prevalence of intestinal parasites	1114 (4%)	91 (31%)
Prevalence of <i>Blastocystis</i> only	604 (2.1%)	42 (14.2%)
Technique used for diagnosis	Almost same technique , stool microscopy and formalin-ethyl acetate concentration	

Both the prevalence of all intestinal parasites and of *Blastocystis* is higher in samples collected from Manisa city than Istanbul. Istanbul is the capital of Turkey and is considered as an urban area. In contrast Manisa is a smaller city, not on the scale of Istanbul and half of the students came from a shanty area and the other half from a wealthy district with an improved infrastructure. In Manisa, students who lived in good conditions also had a lower *Blastocystis* and intestinal parasite prevalence compared with students who lived in a shanty area. In both studies the techniques used are almost the same and the difference in the prevalence of intestinal parasites between the studies is primarily affected by the location or infrastructure of these cities although the comparison of children vs adults could also be important.

In Malaysia, two studies were carried out by Noor Azian *et al.* (2007) and Suresh *et al.* (2001) and showed differences in prevalence of *Blastocystis*.

**Table 1.7** Comparison of *Blastocystis* prevalence in two studies in Malaysia

Authors	Noor Azian et al.(2007)	Suresh et al.(2001)
Type of patient	An aborigine community	Peoples living in flats
Age group	0-70 years	0-80 years
Location	Pahang city	Kuala Lumpur city
No of samples	130	187
Prevalence of intestinal parasites	72.30%	27.90%
Prevalence of <i>Blastocystis</i> only	52.30%	14.90%
Technique used for diagnosis	Trichrome staining was carried out	Direct microscopy using Trichrome, formalin-ether concentration and modified trichrome, stools were also cultured for <i>Blastocystis</i>

In the study by Noor Azian *et al.* (2007), 130 faecal samples were collected from 7 villages in Pos Senderut, an aborigine settlement in Pahang. 68 samples were positive for *Blastocystis* (52.3%) and 94 of the samples showed intestinal parasites (72.3%). In the other study carried out by Suresh *et al.* (2001), the intestinal parasite prevalence was 27.9% whereas *Blastocystis* was found in only 14.9%. I think it is clear the difference in *Blastocystis* prevalence is affected by the type of patient and the enviroment of these people. The first study was carried out in an aboriginal population who lived in houses built on stilts, with bamboo roofs and no electricity, running water or toilets inside their dwellings. In contrast the other groups of people living in blocks of flats have a good hygiene standard. We can see that both the prevalence of intestinal parasites and *Blastocystis* is higher in the aborigine group.

The variation of *Blastocystis* prevalence within these studies was due to various different reasons, including poor socioeconomic conditions, overcrowding, lack of education, and low standard of sanitation and hygiene. These all contribute to a high prevalence of intestinal parasite infections (Rajeswari, 1994).

*Blastocystis* was originally reported as being associated with diarrhoea in the tropics and subtropics (Jelinek *et al.*, 1997). Numerous of studies have pointed out that travel to the

tropics is an important risk factor for infection with *Blastocystis* (Ashford and Atkinson, 1991, Babb and Wagener, 1989, Hahn and Fleischer, 1985). In contrast, Horiki *et al.* (1997) reported that the possibility of travel as a risk factor for infection was still in question. Recent reports have shown that *Blastocystis* infection is common in residents of tropical, subtropical and developing countries (Ashford and Atkinson, 1990; Babcock *et al.*, 1985; (Cegielski *et al.*, 1993); Elmasry *et al.*, 1990; Nimri, 1993; Nimri and Batchoun, 1994; Reinthaler 1988; Torres *et al.*, 1992; Zafar, 1988; Zaki *et al.*, 1991). Refugees, immigrants, and adopted children from developing countries emerge as having a higher prevalence of *Blastocystis* infection than other adults and children who were raised from birth in the same communities (Guglielmetti *et al.*, 1991, Lee, 1991). This conclusion is based on small numbers and it is unclear whether these people had been infected before arrival. It would also be important to know which countries they came from as some countries have higher prevalence of *Blastocystis* than others. Also foreign workers, such as the Peace Corps volunteers, are also at risk of getting the infection when functioning in areas where this parasite is endemic (Herwaldt *et al.*, 2001). On the other hand, Cirioni *et al.* (1999) found that the prevalence of *Blastocystis* was lowest in groups of travellers returning to Italy from developing tropical countries.

It has been reported that *Blastocystis* has a higher prevalence in developing countries (approximately 30 to 50 %: Ashford and Atkinson, 1990, Torres *et al.*, 1992) compared with developed countries (approximately 1.5 to 10 %: Doyle *et al.*, 1990, Logar *et al.*, 1994, Senay and Macpherson, 1990, Sun *et al.*, 1989, Vickerman, 1994), although exceptions do occur (Reinthaler *et al.*, 1988). Within communities, lower socioeconomic groups or those with lower standards of hygiene may show a higher prevalence than the remainder of the community (Libanore *et al.*, 1991), Nimri 1993, Torres 1992). Rayan *et al.* (2010) illustrated



that intestinal parasites are more frequent among rural children compared with urban children, including *Blastocystis*.

Increased risk of infection may also be linked with the workplace. A recent survey in Malaysia by Raja Salim *et al.* (1999) illustrated that people working intimately with animals were at higher risk of getting *Blastocystis* infection. The authors suggested that the animal handlers most likely gained the infection from the animals through the faecal-oral route.

Prevalence of *Blastocystis* does not show large differences between genders (Kain *et al.*, 1987, Garcia *et al.*, 1984, Sun *et al.*, 1989, Torres *et al.*, 1992) although some papers report that the incidence in female adults is slightly higher (Doyle *et al.*, 1990, Kain *et al.*, 1987, Sanad *et al.*, 1991, Senay and Macpherson, 1990, Wang *et al.*, 2002, Nascimento and Moitinho, 2005). Studies of *Blastocystis* infections among children showed that the ratio of male to female is 2:1 (Nimri, 1993; Nimri and Batchoun, 1994) but the significance of this has not been determined.

The occurrence of *Blastocystis* infection has been linked to weather conditions, with the proposal that infections are more common during hot weather (el Masry *et al.*, 1990, Knowles and Gupta, 1924). In contrast, other studies by Cegielski *et al.* (1993), Stenzel and Boreham (1996) and Senay and Macpherson, (1990) did not find any difference in the number of infections at different times of the year. Seasonal studies of human parasite infections in the USA by Amin (2002) showed that the prevalence of *Blastocystis* is highest between July and October then decreases in December. These dates do not contradict a possible summer infection peak. Exposure, experience of symptoms, seeking medical advice, and testing could explain this. The result of Suresh and Smith (2004) was not much different

from Amin (2002) where they found infection more common in summer (July-September) than in winter/spring.

In summer people do go out for picnics and barbecues and there are more eating raw vegetables like salad, cucumber and tomato which could be contaminated with parasites like *Blastocystis*. Also people tend to travel more in summer especially to tropical countries where the hygiene level considered as low and they could get exposure to contaminated food or water either by eating, drinking or even swimming in rivers. In summer people may also go out to farms and petting zoos where they may get exposure to animals known to carry *Blastocystis* like sheep, cow or even birds.

## **1.9 Diagnosis**

### **1.9.1 Smear microscopy**

Diagnosis of *Blastocystis* in laboratories worldwide is achieved mainly by using light microscopy of wet mounts of either fresh stools or concentrates. Permanent faecal smears using the Trichrome staining technique have been recommended for routine use in the diagnosis of *Blastocystis* (Garcia *et al.*, 1984, LeBar *et al.*, 1985, Lee, 1991, Markell and Udkow, 1990), although other stains can be used successfully, like iron haematoxylin (Senay and MacPherson, 1990), Gram (Miller and Minshew, 1988), Giemsa (Ricci *et al.*, 1984) and Wright's stains (Vannatta *et al.*, 1985). However, Zierdt (1991) mentioned that the smears of clinical material stained with Gram stain are not usually successful as *Blastocystis* cells lyse. Many diagnostic laboratories consider the vacuolar form of 5-8  $\mu\text{m}$  to be the standard morphology, however, due to the recent observation of the forms in vivo, it is necessary to

search for the smaller multivacuolar forms in fresh faecal samples otherwise infected patients will be misdiagnosed (Stenzel *et al.*, 1991).

### 1.9.2 Concentration technique

Another way to detect *Blastocystis* is through concentration methods used to detect other protozoa and faecal parasites. Some have reported that centrifugation causes disruption of vacuolar, multivacuolar, and granular forms of the parasite (Millar and Minshew, 1988). Conversely, other authors have described concentration methods to be successful for *Blastocystis* (Aldeen and Hale, 1992, Garcia *et al.*, 1984, LeBar *et al.*, 1985, Qadri *et al.*, 1989, Zierdt, 1991a). Examination of multiple stool specimens is recommended to improve the discovery of low numbers of *Blastocystis* and other intestinal parasites. The organism must be differentiated from leukocytes (Zierdt, 1991a), *Cryptosporidium* spp. and the cysts of other protozoa, particularly those of *Entamoeba histolytica*, *Entamoeba hartmanni* and *Endolimax nana* (Gallagher and Venglarcik, 1985). Comparing the efficiency of microscopy and culture in the identification of infected stools, Kukoscke *et al.* (1990) found that culture had no benefits over microscopy. On the other hand, a study by Leelayoova *et al.* (2002) illustrated that xenic in vitro culture (XIVC) was significantly more sensitive for the detection of *Blastocystis* than the formol ethyl acetate concentration technique (FECT) and direct smear. Suresh and Smith (2004) were able to detect *Blastocystis* in faecal samples by XIVC when there was no parasite detected by FECT. A number of reports have shown that the FECT results in very poor sensitivity for parasite detection (Stensvold *et al.*, 2007a).

### **1.9.3 Culture technique**

Culture of clinical specimens is not recommended as a routine procedure but is beneficial when microscopic diagnosis is uncertain. Although Kukoschke *et al.* (1990) found that using culture for detecting of *Blastocystis* has no advantages compared to wet mount examination, Zaman and Khan (1994) showed that cultures are better than direct microscopy in term of sensitivity. However, Zierdt (1991a) reported that culture of *Blastocystis* cells from stool samples was not successful unless large numbers of parasites were present.

### **1.9.4 Serological test**

Serology has been employed in an effort to recognize patients with *Blastocystis* infections, but with very limited success (Stenzel and Boreham, 1996). A lack of humoral immune response was described by Chen *et al.* (1987).

### **1.9.5 PCR**

Detection of *Blastocystis* in stool samples by microscopy can be insensitive (Leelayoova *et al.*, 2002). This has led to the development of techniques for the isolation of *Blastocystis* and extraction of the DNA necessary for PCR (Leelayoova *et al.*, 2004). Parkar *et al.* (2007) revealed that PCR-based methods were more sensitive than in vitro culture for detection of *Blastocystis* but their published work found that favoured growth of a particular subtype could occur in vitro when the host is infected with more than one subtype. In contrast, Stensvold *et al.* (2007) stated that XIVC has very little or no impact on subtype distribution or variation within a given specimen.

The benefit of PCR for identification of *Blastocystis* is well-known. It can be achieved on a stool that is not totally fresh, and PCR is possible where FECT or culture is not practical because of inadequate material. PCR is almost as fast as FECT and much faster than culture techniques. PCR is recommended for screening for *Blastocystis* infection and for use in prevalence determination (Stensvold *et al.*, 2006). Recently a commercial PCR kit has been available for the detection of *Blastocystis* in the laboratory (Clongen Laboratories, LLC).

In summary, in view of current data, trichrome staining of a direct smear together with stool culture is the best non-molecular approach for diagnosis of *Blastocystis* and PCR amplification of *Blastocystis* DNA from fresh stool or stool culture is a more sensitive alternative to microscopy. The advantage of PCR is that it requires less time (approximately three hours) instead of the 3-4 days required for culture. Although PCR is the most sensitive technique it is not always feasible due to cost, equipment and the need for special training compared to trichrome stain and culture. Combinations of diagnostic tests are going to give more accuracy than one diagnostic test.

## 1.10 Genetic diversity of *Blastocystis*

Both human and animal *Blastocystis* isolates are morphologically similar and have most of the forms described (vacuolar, granular and cyst). No work has been carried out to check if there are any tiny morphological variations. The designation of *Blastocystis* species and the ways in which these species may be recognized have not been settled. Initial data indicated that multiple morphological types of *Blastocystis* can be distinguished in human hosts and this suggested that more than one species of *Blastocystis* infects human (Stenzel and Boreham, 1996).

*Blastocystis* has been detected in a range of vertebrate hosts, including rodents, pigs, cows, birds, reptiles and frogs, and some of these have been considered as zoonotic strains through evidence of genotypic homology to the human isolates (Yoshikawa *et al.*, 1996; Clark, 1997).

*Blastocystis* infections are common in a large variety of mammals and is likely that many undescribed species of *Blastocystis* exist (Clark, 1997). Noel *et al.* (2005) suggested based on DNA sequences that more than one species of *Blastocystis* could infect humans. Furthermore, their findings highlighted the low host specificity of this organism and showed that several types of human *Blastocystis* infection might be of zoonotic origin. Based on limited evidence animal handlers appear to have a significantly higher rate of infection with *Blastocystis* than individuals who do not come in contact with animals (Rajah Salim *et al.*, 1999). However, further investigation of different groups with animals exposure is needed before it can be concluded that some *Blastocystis* infections are zoonotic. Hence, it is valuable to screen for genotypes among *Blastocystis* isolates from animals when evaluating the zoonotic potential for human infection (Abe *et al.*, 2003c).

Even though *Blastocystis* is considered to be pleomorphic in morphology, new species names have been designated for isolates from birds based on morphological features alone (Belova and Kostenko, 1990, cited in Abe *et al.*, 2003a). Other new *Blastocystis* species names have been based on dissimilarity in optimal growth temperature and /or electrophoretic karyotype (Chen *et al.*, 1997b, Noel *et al.*, 2005). Karyotypic diversity between human *Blastocystis* isolates is quite extensive (Carbajal *et al.*, 1997, Ho *et al.*, 1994, Upcroft *et al.*, 1989). Because of this, karyotypic variation among *Blastocystis* isolates from human and non-human hosts is not enough to identify new species (Abe *et al.*, 2003c).

Many researchers have reported that *Blastocystis* shows evidence of great genetic and antigenic heterogeneity. These studies have shown that differences can be detected among isolates from the same host, whereas similarities may exist between isolates from different hosts (Tan, 2004). Studying the relative proportion of different genotypes in different hosts might reveal important epidemiological features, e.g. reservoir hosts and patterns of transmission (Hunter *et al.*, 2004). It is expected that different parasite genotypes may have different reservoirs (Hunter *et al.*, 2004). Direct transmission of *Blastocystis* among humans has been reported (Yoshikawa *et al.*, 2000), but most of the *Blastocystis* infections in humans are believed by some to be potentially of zoonotic origin (Iguchi *et al.*, 2007).

The genetic diversity of *Blastocystis* isolates might be linked to the uncertainty in the role of *Blastocystis* in disease (Clark, 1997, Arisue *et al.*, 2002). For example it is possible that only *Blastocystis* belonging to one subgroup are able to cause disease in humans (Clark, 1997). Since there is no suitable animal model existing for *Blastocystis* infection, the pathogenicity of *Blastocystis* cannot be demonstrated experimentally (Tan *et al.*, 2002). Hence, comparing human *Blastocystis* isolates from clinically symptomatic and asymptomatic patients may show a possible association between certain genotypes and the pathogenic potential of this parasite (Yoshikawa *et al.*, 2004b). However, whether specific genotype(s) of *Blastocystis* can cause symptomatic infections in humans remains is a point of energetic debate (Yan *et al.*, 2007).

## 1.11 *Blastocystis* subtypes

*Blastocystis* shows extensive genetic heterogeneity. At present there are 13 subtypes (ST1-13) that have been identified on the basis of small subunit rRNA gene analysis from mammalian and avian hosts (Noel *et al.*, 2005, Noel *et al.*, 2003, Parkar *et al.*, 2007,

Stensvold *et al.*, 2009, Yoshikawa *et al.*, 2004a). Many *Blastocystis* STs show a broad host range, even though they may not all be of zoonotic significance based on previous studies (Abe, 2004, Parkar *et al.*, 2007, Stensvold *et al.*, 2009, Yan *et al.*, 2007, Yoshikawa *et al.*, 2003).

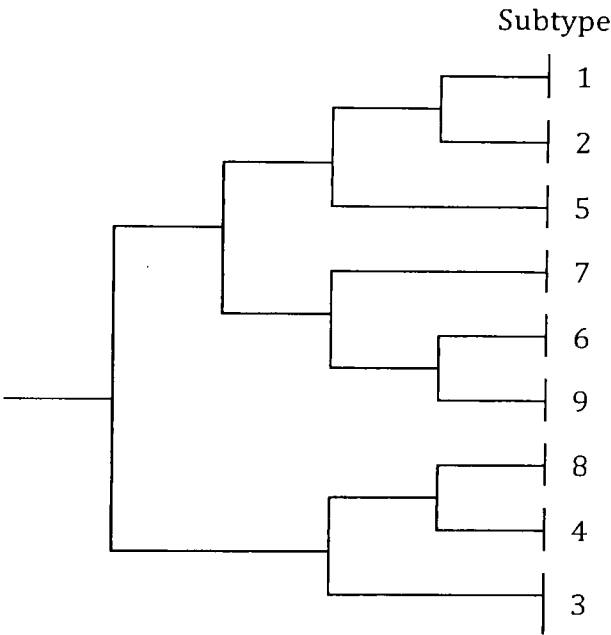


Fig 1.5 Phylogenetic tree showing the relationships among the first nine *Blastocystis* STs

This figure is adapted from Stensvold *et al.* (2007). The original analysis was by Noel *et al.*, (2005) using complete SSU rRNA gene sequences with maximum-likelihood analysis; all of the nodes were supported by 100% in bootstrap analysis.

ST3 has been reported to be the most prevalent subtype in the human population followed by ST1 (Bohm-Gloning *et al.*, 1997, Eroglu and Koltas, 2010, Li *et al.*, 2007, Souppart *et al.*, 2009, Stensvold *et al.*, 2009, Yoshikawa *et al.*, 2004b). STs 1, 2, and 4 have been linked by some to gastrointestinal symptoms (Dominguez-Marquez *et al.*, 2009, Kaneda *et al.*, 2001, Katsarou-Katsari *et al.*, 2008). Others found only ST1 in a group of symptomatic patients (Yan *et al.*, 2006), and Hussein *et al.* (2008) agreed with this hypothesis, proposing that ST1 was linked with increased pathogenicity. In contrast, results from Tan *et al.* (2008) show that



ST3 is linked with disease. Various other studies, however, have indicated there is no correlation between specific subtypes and the pathogenic potential of *Blastocystis* (Bohm-Gloning *et al.*, 1997, Ozyurt *et al.*, 2008, Yoshikawa *et al.*, 2004b)

In non-human hosts, ST1 was the major ST in pigs with a few cases of ST2 (Navarro *et al.*, 2008). According to Yoshikawa *et al.* (2009), ST2 was the higher prevalence subtype in monkeys as well as human. Few cases of ST3 were detected in primate, pigs and cattle by different authors (Abe *et al.*, 2003c, Parkar *et al.*, 2010, Yoshikawa *et al.*, 2004a). To date, ST4 is the only ST found among rodents. This ST has only occasionally been isolated from non-human primates and has not so far been detected in other mammals except an opossum and humans. Overall, in humans ST4 makes up 5% of the isolates (Abe *et al.*, 2003c, Parkar *et al.*, 2010, Stensvold *et al.*, 2009, Yoshikawa *et al.*, 2004a).

A previous survey of *Blastocystis* STs in synanthropic and zoo animals by Stensvold *et al.* (2009) observed that birds usually harbour ST6 and 7 but these are hardly ever found in mammals and have been detected from humans only occasionally. The same authors showed that ST8 was common in non-human primates, especially in woolly monkeys, and it was very rare in humans. ST9 presently consists of only two isolates and these are of human origin (Noel *et al.*, 2005).

ST5 and ST10 have a wide host range, containing isolates from primates, pigs (ST5 only) and other livestock (Noel *et al.*, 2005, Stensvold *et al.*, 2009). Studies of the incidence of *Blastocystis* subtypes in pigs fall in two groups: those that find mainly ST1 (Navarro *et al.*, 2008, Thathaisong *et al.*, 2003) and those that find mainly ST5 (Yan *et al.*, 2007, Yoshikawa *et al.*, 2003, 2004, Stensvold *et al.*, 2009)

Recently three novel subtypes were detected by Parkar *et al.* (2010) from elephant (ST11), giraffe and western grey kangaroo (ST12), and quokka (ST13), a marsupial. They have not yet been reported in other hosts. As many types of animals have not yet been screened for *Blastocystis* subtypes it seems likely that more new STs will be found and wider host ranges will be identified.

The method by which subtyping was performed in Noel *et al.* (2005) paper was using almost full length of SSU rRNA gene sequences of 90 *Blastocystis* isolates from human, rats, birds and reptiles in a large phylogenetic analysis, including some new and some sequences available in databases (Noel *et al.*, 2005). Tree construction was performed using the stramenopile *Proteromonas lacerate*, a commensal flagellate of reptiles and amphibians, as the out-group in view of its position closely related to *Blastocystis* in previous phylogenetic studies.

The data set included 1,563 unambiguously aligned positions, which were analyzed by the maximum-likelihood method using the programme MrBAYES. The rooted tree identified seven clades, each highly supported by Bayesian posterior probabilities (BP) of 100% plus several discrete lineages. Some of these discrete lineages were later called new subtypes by Sciculuna *et al.* (2006) and given the names ST8 and ST9 by Stensvold *et al.* (2007). Many different methods naming subtypes were published by different groups but a consensus naming scheme was produced by Stensvold *et al.* (2007) and this is now used by everyone working on *Blastocystis*.

## 1.12 Multilocus Sequence Typing (MLST)

*Blastocystis* has been divided into numerous subtypes, but the genetic distance between subtypes is significant (up to 7% between SSU rRNA genes) and so they could each be considered distinct species. In other parasites genetic variation within species can sometimes determine virulence and methods to detect variation within species have been developed. The ability to identify precisely the strains of infectious agents that cause disease is a major aim for epidemiological surveillance and public health judgments; however, there are no completely satisfactory methods of achieving this (Maiden *et al.*, 1998)

The most commonly used molecular typing methods, for instance microsatellite typing, pulsed-field gel electrophoresis (PFGE) and arbitrarily primed PCR (AP-PCR), depend on comparison of DNA fragment patterns on gels (Enright and Spratt, 1999). Another typing method is multilocus enzyme electrophoresis (MLEE), which uses protein variation that accumulates very slowly in the population (Maiden *et al.*, 1998). MLEE was the main method for characterising bacterial isolates for epidemiology studies for many years and has given many insights into population structures of bacterial pathogens (Musser *et al.*, 1986). MLEE uses the variation in the electrophoretic mobilities of house-keeping enzyme rather than in the corresponding genes (Spratt, 1999). MLEE has many attractive features and provides data that are useful for population genetic analysis. Although this technique has been used mostly for bacteria it has also been used for *Giardia lamblia* (Meloni *et al.*, 1995), *Trypanosoma cruzi* (Diosque *et al.*, 2003) and *Plasmodium falciparum* (Abderrazak *et al.*, 1999). However, because it relies on cultures it is laborious and generates results that are difficult to compare between laboratories (Spratt, 1999).

More recently, a modification of this method (Multi-locus sequence typing; MLST) has been developed (Spratt, 1999). This has the advantage of the speed and simplicity of DNA sequencing. It is similar to MLEE by studying variation present in multiple housekeeping genes, but with MLST the variation is detected by sequencing gene fragments (Urwin and Maiden, 2003).

Although molecular methods have been used to distinguish between *Blastocystis* subtypes genetically (Stensvold *et al.*, 2008), none exist to detect variation within subtypes. To understand more about transmission, host specificity and pathogenicity of this parasite we need a tool which helps to differentiate between strains of the same subtype and MLST is one of the best methods to carry out this work with the information currently available.

## 1.13 Summary of background information

*Blastocystis* is a prevalent enteric protist that infects a variety of animals and is classified taxonomically within the heterogeneous group, the Stramenopiles, which includes water moulds, slime nets and brown algae. *Blastocystis* was the first Stramenopile known to parasitise humans. Morphologically, *Blastocystis* is polymorphic and there are few main stages that have been described. There are different conflicting life cycles that have been proposed and this inconsistency is due largely to the belief that *Blastocystis* demonstrates multiple reproductive processes. Until confirmed otherwise, binary fission is the only accepted mode of reproduction. Transmission of the parasite is by a cyst stage, considered as the only infective stage, and it is widely accepted that *Blastocystis* is transmitted by the faecal-oral route like other gastrointestinal protozoa. Waterborne transmission of *Blastocystis* through untreated water or poorly sanitary conditions has also been indicated, and person to

person transmission among family members, among mentally deficient persons in institutions and in communities without sufficient sanitary facilities is likely occurring also.

It is unclear whether *Blastocystis* is a true pathogenic organism or a commensal, or possibly is capable of being a pathogen under specific conditions. Many reports suggest that *Blastocystis* causes disease. However, there also have been a similar number of reports to the contrary. Elimination of all other infectious and non-infectious possible causes of symptoms is a major problem, especially when the pathogenicity of many other associated organisms is also uncertain. Many studies that investigate the pathogenicity of *Blastocystis* have not eradicated all known viral, bacterial, or other protozoal infections as possible causes of symptoms while others have not considered non-infectious causes. Some authors have concluded that *Blastocystis* is pathogenic because chemotherapy has resulted in a reduction of symptoms and elimination of organisms. Other authors indicate that *Blastocystis* may cause symptoms when present in large numbers as severity of symptoms has been reported to correlate with the numbers of *Blastocystis* found in faecal samples. At present, the inability to fulfil Koch's postulates, mainly due to a lack of experimental animal models, and difficulty in excluding all other sources of symptoms means the role of *Blastocystis* as a causative agent of human disease remains unclear.

Irritable bowel syndrome (IBS) is a complicated, multifaceted heterogeneous disorder, rather than a single disease. *Blastocystis* has been linked to IBS but the role of *Blastocystis* in IBS is again unclear due to the uncertainty surrounding its pathogenicity. However, symptoms that have been linked to infection with *Blastocystis* are similar to those present in IBS and include diarrhoea, abdominal pain, cramps or discomfort, and nausea. The prevalence of *Blastocystis* in IBS patients has been reported to be higher than in the general population.

The need for treatment of *Blastocystis* infections is controversial in the absence of conclusive evidence of pathogenicity of this organism. There are a few studies investigating the effect of drugs on *Blastocystis* in vitro, with the most effective drugs reported to be metronidazole, trimethoprim sulfamethoxazole, paromomycin, rifaximin and Nitazoxanide. Metronidazole is the most frequently prescribed antibiotic, used alone or in combination with another, such as paromomycin or cotrimoxazole (TMP-SMX).

*Blastocystis* is the most frequently isolated parasite in epidemiological surveys and its prevalence varies widely from one country to another and among communities of the same country. In general, developing countries have higher prevalence of the parasite than developed countries and this has been linked to poor hygiene, exposure to animals and consumption of contaminated food or water.

*Blastocystis* most often has been diagnosed by light microscopic examination of faecal material using wet mounts, either unstained or stained with iodine. Culture from faecal material is reported to have no advantages over light microscopy for the detection of *Blastocystis*. PCR amplification of *Blastocystis* DNA from fresh stools or stool cultures is a more sensitive alternative to microscopy, and genotyping should be included in the analysis where possible.

*Blastocystis* has been isolated from a wide range of hosts including primates, livestock, rodents, birds, reptiles, amphibians and insects. *Blastocystis* demonstrates evidence of great genetic heterogeneity, and differences can be detected among isolates from the same host, whereas similarities may exist between isolates from different hosts. At this time 13 subtypes have been recognized based on small subunit rRNA gene analysis from mammalian and avian hosts.

The genetic distance between subtypes is significant (up to 7%) and they could be considered distinct species. In some parasites, genetic variation within species can determine virulence and methods to detect variation within species have been developed. None have been applied to *Blastocystis* so far. The method Multi-locus sequence typing (MLST) has been reported to have the advantage of speed and simplicity of DNA sequencing, and seems the best choice for studying *Blastocystis* variation within subtypes.

## **AIMS AND OBJECTIVES**

### **1.13.1 Aims**

The aim of this study is to understand the significance of genetic diversity in *Blastocystis*.

### **1.13.2 Objectives**

The objectives of this study are

#### **Objective No 1**

To compare the *Blastocystis* subtype distribution between the UK and Libya

#### **Objective No 2**

To investigate *Blastocystis* subtypes in IBS patients

#### **Objective No 3**

To investigate associations between *Blastocystis* subtypes and specific hosts

#### **Objective No 4**

To investigate the possibility that *Blastocystis* infection is a zoonosis



## **Objective No 5**

To develop a MLST method that distinguishes strains within *Blastocystis* subtypes

Not all the objectives were completely achieved in this study, and each separate objective will be considered in detail in each results chapter.

# CHAPTER 2

## Materials and methods

### 2.1 *Blastocystis* samples

Most of the UK samples from both humans and animals were provided as culture lysates by the Diagnostic Parasitology Laboratory of the London School of Hygiene and Tropical Medicine. The origin of these samples came from IBS clinics, patients referred by a physician for ova and parasite analysis, and different zoo locations. The London School of Hygiene and Tropical Medicine ethics committee approved this study under Reference number 5026. Libyan patients supplied their stool samples to Sebha Central Medical Laboratory for a variety of reasons, for example, gastrointestinal symptoms, applying for a job in the food industry, routine screening prior to army enlistment. The ethics committee of Sebha University in Libya said no approval was needed to carry out this additional work (date of the letter 10/9/2008).

Both Libyan and UK faecal samples were examined by direct microscopy using normal saline solution and Lugol's iodine stain. Approximately 50 mg of faecal sample was inoculated into Robinson's medium (Clark and Diamond, 2002) and incubated at 37 °C. Also a modification of Jones' medium (Leelayoova *et al.*, 2002) using a Luria agar slant and supplemented with 10% human serum was used to culture *Blastocystis* from some human and animal faecal samples in Libya.

The screening for *Blastocystis* in 3-4 day-old cultures was done with standard light microscopy and subculturing was carried out every 2-3 days. *Blastocystis* growing in medium was harvested by centrifugation at 13,000 xg for 10 seconds. Supernatant was discarded carefully and the tube vortexed vigorously to resuspend the cells in the residual supernatant.

For larger preparations, *Blastocystis* cells were transferred into LYSGM medium with 5% adult bovine serum (Stechmann *et al.*, 2008) and then subcultured every 3-4 days until the parasite established in the medium. The whole culture volume (10 ml) was then transferred to a tissue culture flask of LYSGM (50ml) and incubated in order to obtain more material for DNA preparation.

## **2.2 DNA extraction from cultured cells**

### **2.1.1 Cell lysis**

300 µl of cell lysis solution was added to the resuspended cells (ca.50 µl) to lyse the *Blastocystis*. This lysis solution contains 0.25% sodium dodecyl sulfate (SDS), a detergent that helps to break down the cell membrane. 0.1M Ethylenediamine tetraacetic acid (EDTA) pH8 is present to inhibit nucleases by complexing with the divalent cations needed by these enzymes.

### **2.1.2 Purification of DNA**

Puregene core kit A (Qiagen, Crawley, UK) was used for DNA purification according to the manufacturer's protocol. The protocol is presented briefly below.

### **2.1.3 Protein removal**

1.5  $\mu$ l of Proteinase K (20 mg/ml) was added to the 300  $\mu$ l lysate and the mixture was vortexed for 5 seconds at medium speed, followed by incubation at 55 °C for 30 minutes to degrade the proteins.

### **2.1.4 Removal of RNA**

4  $\mu$ l of RNase A (4 mg/ml) was added to the lysate to degrade RNA and incubated at 37 °C for 5 minutes. Then the tube was cooled to room temperature by placing in ice for 1 minute.

### **2.1.5 Protein precipitation**

Protein and other contaminants were precipitated from the cell lysate using a high concentration of salt. 100  $\mu$ l of ammonium acetate (the concentration of ammonium acetate is not given by the manufacturer) was added to the tube, vortexed at high speed for 20 seconds, and centrifuged at 13,000 xg for 1 minute. A tight pellet is formed.

### **2.1.6 DNA precipitation**

The supernatant having the DNA was removed into a fresh 1.5 ml microcentrifuge tube containing 300  $\mu$ l of 100% isopropanol. The sample was inverted gently 50 times to mix and precipitate the DNA, then centrifuged at 13,000 xg for 1 minute. DNA becomes visible as a small white pellet.

The supernatant was discarded and 300µl of 70% ethanol was added, the tube inverted several times to wash the DNA pellet and centrifuged at 13,000 xg for 1 minute. After the supernatant was removed carefully, the tube was left with open lid for 15 minutes to help evaporation of ethanol. 50µl of DNA hydration solution (a low salt buffer) was added and vortexed for 5 seconds at medium speed to break the pellet. The tube was incubated for 5 minutes at 65 °C to accelerate hydration then vortexed for 5 seconds at medium speed to mix the sample. DNA was stored at 4 °C, or -20 °C for long storage.

## **2.3 DNA extraction directly from faecal samples**

Some DNA was extracted directly from Libyan cow faecal samples using a QIAamp DNA Stool Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's protocol.

## **2.4 Polymerase chain reaction**

### **2.4.1 Barcoding region of SSU rRNA gene**

'DNA barcoding' is the term used to describe a method proposed for producing a unique identifier for all living species (*Hebert et al., 2003*). This system is expected to be very beneficial for understanding the diversity of uncultured protozoan lineages that do not have enough morphological details for species separation (*Tautz et al., 2002*). *Herbert et al. (2003)* suggested that a DNA bar coding system for animals could be based on sequence diversity in a 650 bp fragment of the mitochondrial cytochrome C oxidase subunit I (cox1) gene. Although this gene was confirmed to be helpful for the identification of animal species, and also some protists (*Chantangsi et al., 2007*), this gene is not usable for all groups of eukaryotes (*Scicluna et al., 2006*). For example, anaerobic eukaryotes do not normally have a

mitochondrial genome and therefore the gene (cox1) is not present, and many other groups of eukaryotes are yet to be studied at the level of the mitochondrial genome. In addition, a few groups of eukaryotes, such as ciliates, have different sized insertions within the cox1 gene that produces difficulties in the PCR amplification of the barcode sequences (Norman and Gray, 1997).

SSU rRNA genes also contain regions of sequence variation that are useful for DNA barcoding; for instance, Scicluna *et al.* (2006) established the usefulness of a 600 bp segment of SSU rDNA for identifying subtypes of *Blastocystis*. Although it is unusual for an anaerobic organism to have one, the mitochondrial genome present in *Blastocystis* does not have a cox1 gene and so barcoding in *Blastocystis* has focussed on the SSU rDNA method developed by Scicluna *et al.* (2006). This gene is also present in numerous copies within the genome of most eukaryotes, which makes it easy to amplify, and contains highly conserved regions that allow the design of universal primers for PCR amplification (Sogin *et al.*, 1986).

PCR amplification of *Blastocystis* SSU rDNA for 'Barcoding' was performed in a 40 µl volume per reaction using the two primers BhRDr and RD5 (Table 2.1) at 2 µM concentration to amplify a 600 bp region (Scicluna *et al.*, 2006), DNA template, Biomix (Bioline, London, UK) and deionized water. The following amplification profile was used: 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 59 °C for 30 seconds and extension at 72 °C for 30 seconds.

Amplification was performed using a G-storm GS1 thermal cycler (Gene Technologies Corp. Surrey, UK). PCR products were separated by electrophoresis using 1.2% (w/v) agarose gels in Tris-Boric Acid-EDTA buffer. The gel was stained with ethidium bromide and the bands

observed under ultraviolet light and photographed. Specific bands were cut out when required.

### **2.4.2 Complete SSU rRNA gene**

The whole SSU rRNA gene was amplified using the protocol of Clark (1997) which uses the primers RD5 and RD3 (Table 2.1). PCR amplification was performed as above with the following amplification profile: a denaturation step of 94 °C for 4 min then 30 cycles of amplification of 1 minute at 94 °C , 90 seconds at 65 °C, and 2 minutes at 72 °C.

### **2.4.3 PCR product purification**

Two methods for product purification were used. In the first, PCR products were extracted from gel pieces using the QIAquick gel extraction kit according to the manufacturer's protocol (Qiagen, Crawley, UK). Sodium iodide is used to solubilise the gel for 10 minutes at 50 °C until the gel dissolves completely. 100% isopropanol (0.3 ml) is added to the sample and mixed. The solution is then transferred into the spin column, centrifuged for 1 minute, the flow-through discarded and 0.5 ml of Sodium iodide solution added to wash the column. After centrifugation, 0.75 ml of wash buffer PE is added to the column. The purpose of this step is to get rid of all Sodium iodide, and PE contains ethanol to keep DNA bound to the column. After standing for 2-5 minutes, the column is centrifuged for 1 minute then changed to a clean 1.5 ml microcentrifuge tube and 20 µl of sterile deionized water added. The column is allowed to stand for 1-2 minutes to allow the DNA to release from the column then centrifuged for 1 minute at 13,000 xg. The DNA is then ready to be used for sequencing reactions.

GeneJET PCR Purification Kit was also used, according to the manufacturer's protocol (Fermentas, York, UK). A binding buffer is added to the PCR mixture, then transferred to the column and centrifuged for 30-60 seconds at 13,000 xg. The flow through is discarded and the column washed with 700  $\mu$ l wash buffer, centrifuged for 30-60 seconds, the flow through discarded, and the column transferred to clean 1.5  $\mu$ l microcentrifuge tube. 20  $\mu$ l of deionized water is added, the column allowed to stand for 2 minutes then centrifuged for 1 minute at 13,000 xg. Then the DNA is ready to be used.



#### 2.4.4 Barcoding sequence

The sequencing primer used is identical to the internal primer (BhrDr) of the PCR reaction. Either BhrDr or RD5 primer can be used to sequence barcoding region but BhrDr tends to give better quality sequence compared with RD5.

During my work I have tried both RD5 and BhrDR primers and I found that BhrDR primer is given better sequence than RD5. Also Sciculuna *et al.* (2006) reported this difference between these primers where the peaks were higher and background was lower with BhrDR when traces for the two stands were compared for the same sample and they recommended BhrDR for *Blastocystis* DNA barcoding. Therefore, in this study we used BhrDr. 2 µl of PCR product was used for sequencing, along with 1 µl (0.2 µM final concentration) of 1:100 diluted primer, 0.5 µl of Big Dye v.3.1 reaction mix (Applied Biosystems, Warrington, UK), 2 µl 5x sequencing buffer and finally 4.5 µl of sterile deionized water. The total volume is 10 µl. The following reaction profile was used as recommended by Applied Biosystems: 25 cycles of

Step one: denaturation at 94 °C for 15 seconds.

Step two: annealing at 50 °C for 15 seconds.

Step three: extension at 60 °C for 4 minutes.

**2.4.5 Complete SSU rRNA gene sequence**

The same protocol for bar-coding sequence was used except that we used several internal primers (Figure 2.1) (as well as RD3 and RD5) to cover the whole region. Primers were diluted 1:100, at 0.2 µM final concentration. All primers used to sequence the SSU rRNA gene are listed in Table 2.1

**Table 2.1** Primers used for sequencing of complete SSU rRNA genes

Primer	Sequence
RD3	5' TGATCCTTCCGCAGGTTACCTAC 3'
RD5	5' ATCTGGTTGATCCTGCCAGTA 3'
BhrDr	5' GAGCTTTTAACTGCAACAACG 3'
528 R	5' GAGCTGGAATTACCGC 3'
528 F	5' GCGGTAATTCCAGCTC 3'
1055 R	5' ACGGCCATGCACCAC 3'
1055 F	5' GTGGTGCATGGCCGT 3'
1200 F	5' CAGGTCTGTGATGCCC 3'
AEMH5 F	5' TCCAAGGAAGGCAGCAGGC 3'
AEMH3 R	5' AAGGGCATCACGGACCTGTT 3'

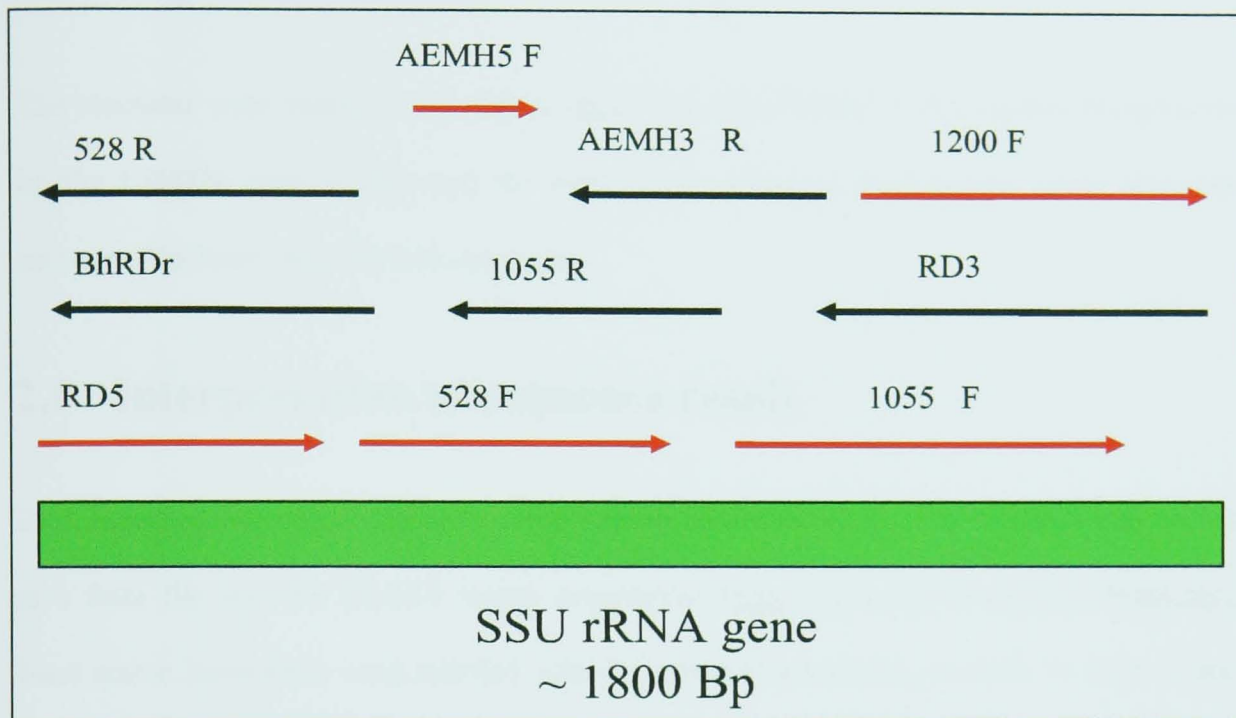


Fig 2.1 Location of the 10 primers used to sequence complete SSU rRNA genes. Red = forward primer, black = reverse primer

## 2.4.6 Precipitation of sequencing products

After the sequencing reaction was completed, 40  $\mu$ l of 75% isopropanol ( $-20^{\circ}\text{C}$ ) was added to precipitate the sequence products, vortexed for a few seconds and incubated at room temperature for 15 minutes, then centrifuged at 13,000  $\times g$  for 20 minutes. The supernatant was discarded with care leaving the DNA at the bottom, followed by adding 180  $\mu$ l of 75% isopropanol ( $-20^{\circ}\text{C}$ ) to wash the pellet, vortexing for 20 seconds and centrifuging at 13,000  $\times g$  for 8 minutes. The purpose of using isopropanol is to precipitate the DNA and to get rid of unused dye-labelled nucleotides. Pellets were dried in a heating block at  $94^{\circ}\text{C}$  for 4 minutes. The sample is now ready for sequence reading.

## 2.5 Sequence reading

The reactions were analyzed on a DNA sequencer ABI PRISM 3730 (Applied Biosystems) by the LSHTM core facility and the output was examined for accuracy using Chromas software (Technelysium Pty Ltd, Australia).

## 2.6 Interpretation of sequence result

Each sequence was edited manually using Chromas software v1.61. The sequence was copied as a fasta file into the BLAST search programme (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Blast search parameters were selected with the option to search for similarity to *Blastocystis* sequences in GenBank. The results were presented with different percentage identity and similarity. The sequence in GenBank with the highest percentage identity, which is usually approximately 98-100%, was considered to identify the subtype of the query sequence. If the top hits observed in the BLAST search were shown to have 97% identity or less, this was considered to perhaps be the result of identifying a new subtype and further investigation to compare it with closely related subtype sequences was performed using Multalin (Corpet, 1988) and phylogenetic analysis to confirm whether it represented a new subtype.

## 2.7 Phylogenetic analysis

Phylogenetic trees were constructed using sequence data from complete gene sequences of the SSU rDNA. Sequences of genes obtained in the present study were aligned with previously published sequences of *Blastocystis* (ST1-ST13) obtained from GenBank using the program BioEdit version 7.0.9.0, and then manually adjusted where necessary. Phylogenetic analysis was performed using MEGA 5.0 (Tamura et al., 2011). Trees were

constructed using both neighbour-joining and maximum likelihood methods. For the neighbour-joining tree bootstrap values were calculated by the analysis of 1000 replicates. The options for the analysis were pairwise deletion for gaps and maximum composite likelihood, heterogeneous pattern among lineages and different rate among site as the model. The gamma parameter is 0.3. In maximum likelihood bootstraps values were calculated using 100 replicates. The mode used was General Time Reversible with gamma distribution and Invariant sites, and all sites were used. This model was chosen as best by Model Test in MEGA 5.

*Proteromonas lacertae* was used as the outgroup because it is the closest known relative to *Blastocystis*. The two methods used will be described briefly:

### **2.7.1 Neighbour-joining (NJ)**

**Neighbour-joining (NJ)** is a tree-building process that uses the distance approach where the distance between each pair of sequences is used to estimate the best tree. This method also does not always produce the tree that best fits the data, but computer simulations have shown that is quite efficient in obtaining the correct tree. It can be used with any type of distance data (Saitou and Nei, 1987) and one of the advantages of the neighbour-joining method is that it is quite easy and quick to perform, largely because the multiple alignment has been reduced to a simple form- a table of pairwise distances between sequences.

### **2.7.2 Maximum likelihood (ML)**

Maximum likelihood (ML) evaluation has been widely accepted as one of the most accurate techniques for reconstructing phylogenies.

Felsenstein (1981) first applied this method to nucleotide-based phylogenetic analyses. Numerous computer studies (Huelsenbeck, 1995, Kuhner and Felsenstein, 1994, Rosenberg and Kumar, 2001, Ranwez and Gascuel, 2002) have shown that ML programmes can recover the correct tree from artificial data sets more often than other methods. The maximum likelihood method estimates the correct tree by finding the tree with the highest probability of occurring, this depends on using estimates of the mutation rate and other parameters that are either obtained from other studies or estimated from the data.

The advantage of maximum likelihood is that the evolutionary model used can include other variables thought to be biologically important. However, the more complex the evolutionary model is the more computer intensive and time-consuming the phylogenetic analysis becomes.

Other types of methods like maximum parsimony and UPGMA are not widely used compared to ML and NJ.

### **2.7.3 Bootstrap analysis**

Bootstrapping is a common approach used to make statistical conclusions about phylogenetic trees. The term “bootstrapping” was defined by Efron (1979) as “to obtain estimates of error in nonstandard situations by resampling the data set many times to provide a distribution against which hypotheses can be tested”. Very soon after the introduction of the bootstrap Felsenstein (1985) proposed using bootstrapping as a method for obtaining confidence limits on molecular phylogenies. For sequence data, positions in the multiple alignment are resampled and a new best tree calculated. This is repeated many times.

The proportion of samples in which a clade is recovered is indicated as a percentage and referred to variably as the bootstrap value or bootstrap percentage (BP).

The percentage is usually indicated on the tree figure next to the relevant clade. The higher the value obtained, the greater the confidence we have in the grouping indicated by that node in the tree. Because each re-sampling involves calculating the best tree from that data set, bootstrapping adds a lot of time to a phylogenetic analysis so the number of re-sampled data sets used is sometimes determined by the time and computing power available.

## **2.8 Development of Multilocus sequence typing (MLST)**

Specific details involved in the development of the MLST method will be given in chapter 5.

## **2.9 Statistical analysis**

Statistical analysis was performed using a statistical web site:

<http://faculty.vassar.edu/lowry/VassarStats.html>

Chi-square test was used to assess the significance of relationships between *Blastocystis* subtypes and hosts. Probability values of less than 0.05 were considered statistically significant.

## **Chapter 3**

# **Distribution of *Blastocystis* subtypes in UK and Libya populations**



### 3.1 Introduction

*Blastocystis* is a ubiquitous parasite with a worldwide distribution (Jelinek *et al.*, 1997, Tan, 2004). It is common for it to be the most frequently isolated parasite in epidemiological surveys (Amin, 2002, Baldo *et al.*, 2006, Taamasri *et al.*, 2000). Incidence varies from country to country and within various communities of the same country. Generally, developing countries have a higher frequency of the parasite than developed countries and this has been related to poor hygiene, exposure to animals and consumption of contaminated food or water (Tan, 2008).

DNA- based methods are able to identify genetic variation between *Blastocystis* organisms which seem to be morphologically similar. In spite of these recent methodological advances, few studies have investigated the molecular epidemiology of *Blastocystis* infections. Such studies have mostly focused on the difference in incidence of *Blastocystis* subtypes between asymptomatic and symptomatic individuals (Bohm-Gloming *et al.*, 1997, Kaneda *et al.*, 2001; Yoshikawa *et al.*, 2004; Yan *et al.*, 2006; Souppart *et al.*, 2009). Recent surveys have included subtype information obtained by PCR of *Blastocystis* DNA directly from faeces or stool culture, and such studies are starting to provide more information on the distributions of subtypes among human populations (Tan, 2008).

Several studies of *Blastocystis* subtypes in humans were carried out in temperate countries like UK, France, Germany, Italy and USA. The Libyan climate is different from these countries especially in the south where there is dry hot weather most of the year. We decided to investigate whether there is any difference in the distribution of *Blastocystis* subtypes between temperate and hot climate countries, and to expand the number of samples in UK to

compare it with other studies. In addition, we wanted to compare Libyan *Blastocystis* subtypes with those in Egypt, which has long border with Libya, as a report showed a high prevalence of unusual subtypes like ST6 and ST7 (Hussein *et al.*, 2008) which are considered as rare subtypes in humans worldwide.

In Libya, *Blastocystis* subtypes have not been investigated previously and the present study aimed to characterize *Blastocystis* isolates from unselected Libyan people in order to compare the Libyan subtype distribution with the UK population and that seen in other countries.

## **3.2 Materials and Methods**

### **3.2.1 Source of specimens**

A total of 147 stool samples were used in this study from both the UK and Libyan unselected populations. In the UK, the GP asks for ova and parasite examination usually because patients are symptomatic but the cause is unknown. In Libya, samples are sent by physician for ova and parasite examination in order for the patient to get a health certificate, to join the army or for a student to join the university.

#### **3.2.1.1 UK sample**

Culture lysates for one hundred and nine faecal specimens from GP submissions between 2004-2010 for ova and parasite diagnosis were obtained from the Diagnostic Parasitology Laboratory in the London School of Hygiene and Tropical Medicine.

### **3.2.1.2 Libyan samples**

Both the Mediterranean Sea and the desert affect Libya's climate. In the winter, the weather is cool with some rain on the coast and in the drier desert the temperature can drop to sub-freezing at night.

The Sahara is basically very dry and hot in the summer and cool and dry in the winter. Temperatures in the summer can reach 50°C during the day but more commonly are around 40°C. Summer night temperatures can vary from 30 to 40°C.

Sebha is a city in southwestern Libya (Fig 3.1) at Latitude 27.0333 Longitude 14.4333 with a population of 130,000. It was historically the capital of the Fezzan region and is now capital of the Sebha District. The weather is hot and dry in summer and cold and dry in winter.

Thirty-eight faecal samples were collected from unselected Libyan patients attending Sebha Central Medical Laboratory for ova and parasite examination.



Fig 3.1 Map of Libya showing the location of sample collection from both humans and animal hosts

### 3.2.2 Sample processing

For isolation of *Blastocystis* parasites, DNA extraction, PCR and sequencing see chapter 2

sections 2.1-2.7 for details

## 3.3 Results

All of the samples included in this study represented single subtype infections and seven subtypes have been identified by barcoding. One hundred forty seven isolates were investigated in total, as summarized in Table 3.1.

ST3 was the most common subtype in UK population (45%) followed by ST4 (23%), ST1 (17%) and ST2 (10%). Subtypes 5, 7 and 8 were detected in a low percentage of samples in this population. Only four STs were identified within the Libyan population. ST1 was the dominant subtype with 50% followed by ST3 (40%). ST2 (8%) and ST7 (2.6%) were the only others detected.

**Table 3.1** Numbers of each subtype in UK and Libya random groups

Country	Subtype								
	ST1	ST2	ST3	ST4	ST5	ST6	ST7	ST8	Total
UK	19 (17%)	11 (10%)	49 (45%)	25 (23%)	2 (2%)	—	2 (2%)	1 (1%)	109
Libya	19 (50%)	3 (8%)	15 (40%)	—	—	—	1 (3%)	—	38
Total	38 (26%)	14 (6%)	64 (44%)	25 (17%)	2 (1.4%)	—	3 (2%)	1 (0.7%)	147

Statistical analysis of the data was performed for all pairs except that the numbers for STs 5-8 were combined. The Chi-square test gave a P value of 0.0003 so the difference between UK and Libya subtype distribution is highly significant. It is clear that the major differences between the populations are the absence of ST4 and the much higher prevalence of ST1 in Libya. The other subtypes show similar frequencies.

### 3.4 Discussion

To date there are only nine *Blastocystis* subtypes that have been detected in humans worldwide (Stensvold *et al.*, 2009). All previous studies except Motazedian *et al.* (2008) reported that a big majority of human *Blastocystis* infections were attributable to ST3 isolates. The proportions of other subtypes vary between places. ST1 was the third most common variant in the UK, Malaysia and Denmark, while ST2 is the fourth subtype in Germany, UK, Japan, Malaysia and Denmark. ST4 is the second most common subtype in Denmark, Malaysia and commonly found in the UK (Table 3.2). The subtype distribution in the UK population in the present study was quite similar to that found in Europe and North America (Table 3.2) but the Libyan population shows most similarity with the Iranian study as both show ST1 as the most common subtype found (Table 3.2).

Although Libya has a long border with Egypt and people frequently migrate between the two countries still they can show different *Blastocystis* subtype distributions.

Two studies were carried out in Egypt, one by Hussein *et al.* (2008) and the other by Souppart *et al.* (2010). Comparing my data from Libya with Hussein *et al.* (2008) using the chi square test, the P value was 0.0154 which shows a significant difference between the groups. ST3 was the most common ST in Egypt patients whereas ST1 was the most common ST in Libyan patients. ST6 was detected in only in Egyptian patients whereas ST7 was detected in both populations; however in Egypt ST7 was three times more common than in Libyan patients. ST2 was found only in Libyan patients.

Comparing my data with Souppart *et al.* (2010) study revealed that there is a range of subtypes in both populations (Table 3.2) except that ST7 was detected in Libyan patients

only. Comparing the two populations using Chi square, the P value was 0.0293 which showed that there is a significant difference between the populations. This difference is due to the higher ST1 in Libyan patients compared to Egyptian and a higher frequency of ST3 in Egyptian patients. In this study, the Libyan population shows the highest prevalence of ST1 reported worldwide (50%) (Table 3.1) but ST3 prevalence was quite similar to that found in other countries (40%).

The study by Hussein *et al.* (2008) used STS (sequence-tagged sites) for identifying subtypes compared to sequencing in the study by Souppart *et al.* (2010) and in my study. ST5 relies on a panel of subtype-specific primers for PCR to identify subtypes. It is not clear whether this has affected the results in any way, but I think sequencing should be used to identify *Blastocystis* STs in epidemiological studies. The sample number has to be greater than the one used in these three studies, with identification of both age and gender of the patients presented as this might give some clues about the epidemiology of *Blastocystis* STs in different countries.

Because data are limited in number and geography we do not know whether these findings in Sebha are representative of the whole of Libya as the two studies from Egypt showed that they have different results in different parts of the country.

Concerning ST1, it has been suggested by some authors that it might be linked to zoonotic transmission from farm animals (Noel *et al.*, 2005; Tan 2008). The finding that this ST was very common in our Libyan population suggested that contamination from mammals might be significant source of transmission. Many people in Libya keep animals at home, like goat and sheep, or in a family farm, like cow, and camel, and they use them for food and milk. There is more likely to be exposure to animals in Libya than into UK but due to the low

prevalence of ST1 in animals in Libya (see chapter 5) it is unlikely they are the source of infection for humans. In Libya most people have contact with sheep on Scarify Day where Muslim people slaughter hundreds of thousands of sheep every year. People are in contact with sheep during the slaughter or cleaning of the sheep intestines and abdomen for eating. If ST1 is of zoonotic origin the sheep will be the most likely source of transmission but unfortunately we were unable to survey sheep in our study due to availability of time.

None of the previous studies have detected ST8 in humans except Scicluna *et al.* (2006) and Stensvold *et al.* (2009) and they reported 5% (including zoo keepers) and 1% prevalence respectively; in this study ST8 was found only once in the UK (0.7%) (Table 3.1).

ST5 was originally considered to be specific to pigs and cattle (Noel *et al.*, 2005), however recently Parkar *et al.* (2007) and Yan *et al.* (2007) reported occasional ST5 in human isolates. In this study, ST5 was identified only in the UK population and was rare (2%) compared with other subtypes in the same population. Because ST5, ST7 and ST8 are more commonly associated with non-human hosts (see chapter 5) it is possible that these infections originate from contact with livestock, birds and non-human primates, respectively. Information on animal contact by these individuals is not available but in the design of a future study then information about subject occupation has to be obtained; for example, veterinary practitioner, zookeeper, working in animal slaughterhouse, working in an animal market, or any other contact with animals. In addition, the type of house may be important: are they living in a house, flat or shanty house, or in a farm that has animals living there as well. All this information is essential to know if there is likely to be any contact with animals or not.



On the other hand ST5 was not detected in any Libyan human samples but is common in both camels and cows (Chapter 5), therefore, ST5 in humans is not always correlated with prevalence in domestic mammals.

Both Libya and the UK populations carry several subtypes and there were four subtypes in common (ST1, ST2, ST3 and ST7); ST1 shows the high frequency in Libyan samples while ST3 is the most common subtype in UK. ST4, ST5 and ST8 were detected only in the UK samples. ST3 is the second dominant ST in Libya whereas ST4 is the second most common ST in UK. ST6 was not detected in any populations while ST2, ST3 and ST7 showed similar percentages in both groups.

We can observe from Table 3.2 that ST4 is more limited to temperate countries in Europe and North America with the exception of Japan, Malaysia and China. In other countries where ST4 was not detected, ST1 can be considered as high or moderate in prevalence. It is not clear why ST4 is absent from countries like Libya, Egypt, Iran, Nepal and Turkey. It could be due to climate as most temperate countries have ST4 with a few exceptions. Also culture might be playing some role as Libya, Egypt, Iran and some parts of China are considered to be Muslim. These need further investigation.

**Table 3.2** Subtype classification of human *Blastocystis* sp. Isolates from different countries

Reference	Country	No of samples	ST1	ST2	ST3	ST4	*ST5	ST6	ST7	ST8	ST9	Mix ST	Unknown ST
Bohm-Gloning et al. 1997	Germany	78	18 (23 %)	1 (1.3 %)	51 (65.4 %)	5 (6.4 %)	—	—	—	—	—	—	3 (3.8 %)
Clark 1997	UK	29	2 (6.9 %)	1 (3.4 %)	22 (75.9 %)	4 (13.8 %)	—	—	—	—	—	—	—
Yoshikawa et al. 2000	Japan	32	1 (3.1 %)	—	30 (93.8 %)	—	—	1 (3.1 %)	—	—	—	—	—
Kaneda et al. 2001	Japan	55	11 (20 %)	12 (21.8 %)	24 (46.6 %)	6 (10.9 %)	—	2 (3.6 %)	—	—	—	—	—
Thathaisong et al. 2003	Thailand	153	7 (4.6%)	—	138 (90.2%)	—	—	2 (1.3%)	—	—	—	6 (3.9%)	—
Yoshikawa et al. 2004	Japan	50	4 (8%)	—	26 (52%)	2 (4%)	—	11 (22%)	5 (10%)	—	2 (4%)	—	—
	Bangladesh	26	2 (7.7%)	—	24 (92%)	—	—	—	—	—	—	—	—
	Pakistan	10	2 (20%)	—	7 (70%)	—	—	1 (10%)	—	—	—	—	—
	Germany	12	3 (25%)	2 (16.7%)	5 (41.7%)	2 (16.7%)	—	—	—	—	—	—	—
	Thailand	4	1 (25%)	—	1 (25%)	—	—	1 (25%)	—	—	—	1 (25%)	—
Stensvold et al. 2006	Denmark	29	1 (3.4 %)	6 (20.7 %)	15 (51.7 %)	7 (24.1 %)	—	—	—	—	—	—	—
Scicluna et al. 2006#	UK	49	2 (4 %)	8 (16.3 %)	20 (40.8 %)	16 (32.7 %)	—	—	1 (2 %)	1 (2 %)	—	1 (2 %)	—
Yan et al. 2006	China	35	13 (37.1 %)	—	14 (40 %)	—	—	—	2 (5.7 %)	—	—	6 (17.1 %)	—
Stensvold et al. 2007	Denmark	28	5 (17.9 %)	9 (32.1 %)	13 (46.4 %)	1 (3.6 %)	—	—	—	—	—	—	—
Menounos et al. 2007	Greece	45	9 (20 %)	6 (13.3 %)	27 (60 %)	1 (2.2 %)	—	1 (2.2 %)	1 (2.2 %)	—	—	—	—
Li et al. 2007	China	192	47 (24.5 %)	9 (4.7 %)	116 (60.4 %)	1 (0.5 %)	—	1 (0.5 %)	—	—	—	10 (5.2 %)	8 (4.1%)
Hussein et al. 2008	Egypt	44	8 (18.2 %)	—	24 (54.5 %)	—	—	8 (18.2 %)	4 (9.1 %)	—	—	—	—
Motazedian et al. 2008	Iran	45	20 (44.4 %)	4 (8.9 %)	16 (35.6 %)	—	—	2 (4.4 %)	3 (6.7 %)	—	—	—	—
özyurt et al. 2008	Turkey	87	8 (9.2 %)	12 (13.8 %)	66 (75.9 %)	1 (1.2 %)	—	—	—	—	—	—	—
Rivera. 2008	Philippines	12	4 (33.3 %)	2 (16.7 %)	5 (41.7 %)	—	—	1 (8.3 %)	—	—	—	—	—
Wong et al. 2008	Singapore	14	2 (14.3 %)	—	7 (50 %)	1 (7%)	—	—	4 (28.6%)	—	—	—	—
Tan et al. 2008	Malaysia	20	9 (45%)	1 (5%)	10 (50%)	—	—	—	—	—	—	—	—
Dogruman-Al et al. 2008	Turkey	92	17 (18.5%)	20 (21.7%)	51 (55.4%)	—	—	—	—	—	—	4 (4.3%)	—
Souppart et al. 2009	France	40	8 (20 %)	4 (10 %)	20 (50 %)	4 (10 %)	—	—	1 (2.5 %)	—	—	3 (7.5 %)	—
Yoshikawa et al. 2009	Nepal	20	4 (20 %)	4 (20 %)	12 (60 %)	—	—	—	—	—	—	—	—
Tan et al. 2009	Malaysia	40	5 (12.5 %)	—	20 (50 %)	—	—	11 (27.5%)	2 (5%)	—	—	—	2 (5%)
Dogruman-Al et al. 2009	Turkey	35	1 (2.9 %)	10 (28.6 %)	21 (60 %)	—	—	—	—	—	—	3 (8.6 %)	—
Stensvold et al. 2009	Denmark	24	2 (8.3%)	3 (12.5%)	6 (25%)	9 (37.5%)	—	—	—	—	1 (4.2%)	3 (12.5%)	—
Rene et al. 2009	Denmark	99	20 (20.2 %)	15 (15.2 %)	39 (39.4 %)	16 (16.1 %)	—	1 (1 %)	—	1 (1 %)	—	7 (7 %)	—
Souppart et al. 2010	Egypt	20	3 (15 %)	4 (20 %)	12 (60 %)	—	—	—	—	—	—	1 (5 %)	—
Eroglu and Koltas 2010	Turkey	25	9 (36 %)	6 (24 %)	10 (40 %)	—	—	—	—	—	—	—	—
Santin et al. 2011	USA	14	4 (28.6 %)	1 (7.1 %)	3 (21.4 %)	2 (14.3 %)	—	—	—	—	—	4 (28.6 %)	—
Stensvold et al. 2011	Denmark	22	9 (41%)	11 (50%)	—	—	—	—	—	—	—	2 (9%)	—
Meloni et al. 2011	Italy	30	2 (6.7 %)	5 (16.7)	13 (43.3 %)	6 (20 %)	—	—	—	—	—	4 (13.3)	—
Present study	UK	109	19 (17.4 %)	11 (10.1 %)	49 (45 %)	25 (22.9 %)	2 (1.8 %)	—	2 (1.8 %)	1 (0.9 %)	—	—	—
Present study	Libya	38	19 (50 %)	3 (7.9 %)	15 (39.5 %)	—	—	—	1 (2.6 %)	—	—	—	—
<b>Total</b>	<b>All countries</b>	<b>1657</b>	<b>302(18.2%)</b>	<b>170 (11.3%)</b>	<b>933 (56.3%)</b>	<b>109 (7.2%)</b>	<b>2 (0.13%)</b>	<b>43(2.6%)</b>	<b>26 (1.7%)</b>	<b>3 (0.2%)</b>	<b>3 (0.2%)</b>	<b>54(3.3%)</b>	<b>13 (0.8%)</b>

\*Parker *et al.* (2007) also detected one case of ST5 in a human from Thailand, but as the number of samples was small (5) we did not include that study in this table

# 7 samples from zoo keepers were not included in this table from Scicluna *et al.* (2006)

## **The objective**

### **To compare the *Blastocystis* subtypes distribution between the UK and Libya**

The geography of the UK and Libya is very different. One is located in Europe and the other in North Africa, with completely different weather, life style and even diet. Hence investigation of whether there are any differences in distribution of *Blastocystis* subtypes between these countries could give insight into the importance of environment at factors. Samples were collected from random individuals in Sebha city, which is located in the South West of Libya and where the weather almost always dry hot in summer and dry and cold in winter whereas in London the weather is considered as temperate. The results showed that there is a difference in *Blastocystis* ST distribution between the UK and Libya. ST 3 was the most common in the UK while ST1 was the most frequent ST found in Libya. ST4 was the second most common ST detected in the UK whereas in Libya no ST4 was identified. Libya showed a similarity in *Blastocystis* distribution with Iran except that no ST6 was found in Libya but was present in Iran (4.4%). The paper by Santin *et al.* (2011) included a small number of human samples collected from Colombia and when compared to Libya and Iran the subtypes showed a similar distribution, with only of *Blastocystis* ST1, ST2 and ST3. Whether there is any a common factor linking these countries is not clear. In contrast the STs detected in the UK showed a similar distribution to other Europe countries, including Denmark and Italy.

### **Caveats to data interpretations**

The number of samples from the Libyan population is small compared with the UK population. If the numbers were equal then there might be more subtypes detected in the Libyan population like ST4, ST5 or even ST8. Also samples collected from Sebha city may not reflect the whole population of Libya and the same applies to the UK samples. For example more samples should be collected from Tripoli (North West of Libya) and from Benghazi (North East of Libya) and compared with Sebha samples (South West of Libya), and similarly in the UK where samples could be collected from different regions. This could show more STs or a different ST distribution in region of each country and the ST prevalence presented in Table 3.1 could be quite different. In addition, we do not know whether people in either country had been exposed to animals, and this could influence the interpretation.

## **Chapter 4**

### ***Blastocystis* subtypes in IBS patients and unselected hosts**

## 4.1 Introduction

The pathogenic role of *Blastocystis* in the human intestine is questionable because the organism has been found in both symptomatic and asymptomatic persons (Yakoob *et al.*, 2004). Although several reports have suggested that *Blastocystis* causes gastrointestinal disorders, the specific pathogenicity of this organism has not yet been defined. The parasite is commonly associated with gastrointestinal symptoms such as watery and mucous diarrhea, vomiting, abdominal cramps and bloating (Kaya *et al.* 2007). Epidemiological studies also suggest a role in irritable bowel syndrome (Stark *et al.*, 2007, Ustun and Turgay, 2006).

Irritable bowel syndrome (IBS) is a bowel disorder in which symptoms are associated with alteration in the consistency or frequency of stools. Diagnosis of IBS by physicians is carried out using symptom –based criteria known as the Rome criteria. Because *Blastocystis* infection is more common in IBS patients and because symptoms linked to *Blastocystis* resemble IBS, the potential role of *Blastocystis* in IBS needs to be investigated. To see whether there is any link between *Blastocystis* subtypes and irritable bowel syndrome (IBS), I have compared the frequency of subtypes of *Blastocystis* in patients samples submitted from IBS clinics with those patients from GP clinics.

## 4.2 Material and Method

### 4.2.1 Source of specimens

A total of 225 *Blastocystis* isolates were used in this study.

#### **4.2.1.1 IBS samples**

One hundred and sixteen *Blastocystis* -positive samples submitted for routine ova and parasite analysis came from IBS clinics in England.

#### **4.2.1.2 Unselected samples**

109 *Blastocystis*-positive samples submitted by doctors for individual patients were used as the non-IBS control. This is the same group of samples described and analysed in Chapter 3

Both sample groups were received through the Diagnostic Parasitology Laboratory of the London School of Hygiene and Tropical Medicine. The final diagnosis is not known for these patients so it is possible that some unselected individuals were later diagnosed with IBS, and some IBS clinic patients were later diagnosed with other causes of their symptoms.

### **4.2.2 Culture, DNA extraction, PCR and Sequence reaction**

These methods were as described previously. See Chapter 2, section 2.1-2.7 for details.

## **4.3 Results**

Samples were received from 225 individuals, 116 samples from IBS clinic submission unselected group and ST6 only in IBS but ST7 and ST8 were found in both IBS and unselected groups with low frequency (Table 4.1). Although ST3 has the highest frequency in the IBS group (40%), comparing both groups shows that ST4 in IBS is much higher than in unselected. ST1 and ST2 showed similar prevalence in both groups. and 109 samples from GP

submission (Table 4.1). DNA was extracted and subtypes were identified by ‘barcoding’. Eight subtypes were detected in this work and ST3 shows the highest frequency over all. In the IBS samples, ST4 shows the second highest percentage (38%), nearly twice as high as in the ‘control’ group (23%). ST5 was detected only in the

**Table 4.1** Numbers of each sequence type obtained from each group

Host	Subtype								Total
	ST1	ST2	ST3	ST4	ST5	ST6	ST7	ST8	
IBS	13 (11 %)	8 (7%)	46 (40%)	44 (38%)	—	1 (1 %)	1 (1 %)	3 (3 %)	116
Unselected	19 (17 %)	11 (10%)	49 (45%)	25 (23%)	2 (2 %)	—	2 (2 %)	1 ( 1 %)	109
Total	32 (14 %)	19 (8.4%)	95 (42%)	69 (30.7%)	2 (0.9%)	1 (0.4%)	3 (1.3%)	4 (1.8%)	225

The result of chi square test showed the P value is 0.136. The difference in subtype distribution between IBS and unselected samples is not significant.

### 4.4 Discussion

I would like to start by discussing the evidence for *Blastocystis* being associated with IBS. In a study reported by Yakoob *et al.* (2004) at the Aga Khan University Hospital in Karachi, Pakistan, 95 patients with symptoms diagnosed as IBS according to Roma II criteria who attended the gastroenterology clinic from January 2002 to June 2003 were studied. Single stool samples were used and wet mount smears prepared using physiologic saline and Lugol’s iodine. For culturing *Blastocystis*, Jones medium was used without adding starch and the cultures were incubated at 37 °C and examined after 24, 48, 72 and 96 hours.

The authors compared their IBS group with a control group. Age groups were almost the same between IBS and control, being a little higher in the control group, whereas gender showed more males in IBS and more females in the control group. The bowel habit of the control group was described as diarrhoea with loose consistency of stools, which I think is



not good control group as some may have had IBS and they should have used a control group without any diarrhoea symptoms.

The authors compared diagnostic data from both the IBS and control groups using stool microscopy and culture but the data were combined in one table (Table 4.2), which made it unclear for the reader. It would have been clear to separate the data into two comparisons as follows (Table 4.2 & 4.3).

**Table 4.2** Comparison of stool microscopy between IBS and control group

	IBS	Control	Total
Positive	30	4	34
Negative	65	51	116
Total	95	55	150

P=0.005

**Table 4.3** Comparison of stool culture between IBS and control group

	IBS	Control	Total
Positive	44	4	48
Negative	51	51	102
Total	95	55	150

P= < 0.001

From the tables we can observe that both methods show a higher prevalence of *Blastocystis* in IBS patients with high significance. I believe the author’s conclusion that stool culture is more sensitive than stool microscopy but comparing the IBS patients with this control group I

think it is not a good comparison, as this control group is suffering from symptoms like diarrhoea which may bias the result.

In Italy, Giacometti *et al.* (1999) compared the prevalence of *Blastocystis* in patients affected by non IBS gastrointestinal disorders and patients affected by IBS. Over a 2-year period from March 1996 till April 1998, 388 successive individuals with gastrointestinal symptoms were referred to the institute (Table 4.4). 212 individuals were male and 176 were female. Patient ages varied from 18 to 84 years with an average of 44.2 years.

The patients were classified into two groups according to the Roma diagnostic criteria; individuals affected with the functional gastrointestinal disorder IBS (R+) and individuals with gastrointestinal disorders other than IBS (R-). Three samples were collected from each patient and all samples were examined by a direct smear microscopy using Lugol's iodine, then stools were concentrated by a formalin-ethylacetate technique. More than one slide was prepared for detection of ova and parasites.

The authors decided to use the criterion of 5 organisms or more per field to consider someone as positive for *Blastocystis*.

The p value was 0.006 and showed that *Blastocystis* is more common in IBS with high significance (Table 4.5). However, when they compared prevalence of < 5 organisms per field in both groups the p value was 0.085 which is not significant.

**Table 4.4** Prevalence and concentration of *Blastocystis hominis* organisms in the stool of patients with or without irritable bowel syndrome (Taken from Giacometti *et al.* 1999)

Presence of <i>B.hominis</i> (per x 40 field)	No . (%)		P value
	R-patients <sup>a</sup> (n= 307)	R+ patients <sup>b</sup> (n= 81)	
No organism present	284 (92.5)	66 (81.5)	0.006
Any organism present	23 (7.5)	15 (18.5)	
< 5 organism per field			
<i>B.hominis</i> only	13(4.2)	8(9.9)	0.085
<i>B.hominis</i> plus other nonpathogens	4 (1.3)	1 (1.2)	0.613
≥ 5 organisms per field	6 (2.0)	6(7.4)	0.031

<sup>a</sup> Patients affected by gastrointestinal disorders other than irritable bowel syndrome

<sup>b</sup> Patients defined as affected by irritable bowel syndrome according to Rome diagnosis criteria

81 IBS patients were described for their first comparisons. 72 patients reported for follow up at 6 months and out of these 72 patients 53 were no longer considered to be patients affected by IBS and only 19 patients as affected by IBS (Fig 4.1). It is well known that IBS is chronic disease so it seems that some IBS patients may have been misdiagnosed initially and were considered to be IBS patients when in fact they were not. This makes the results not suitable for comparison and I think this can be considered to bias the result in this paper.

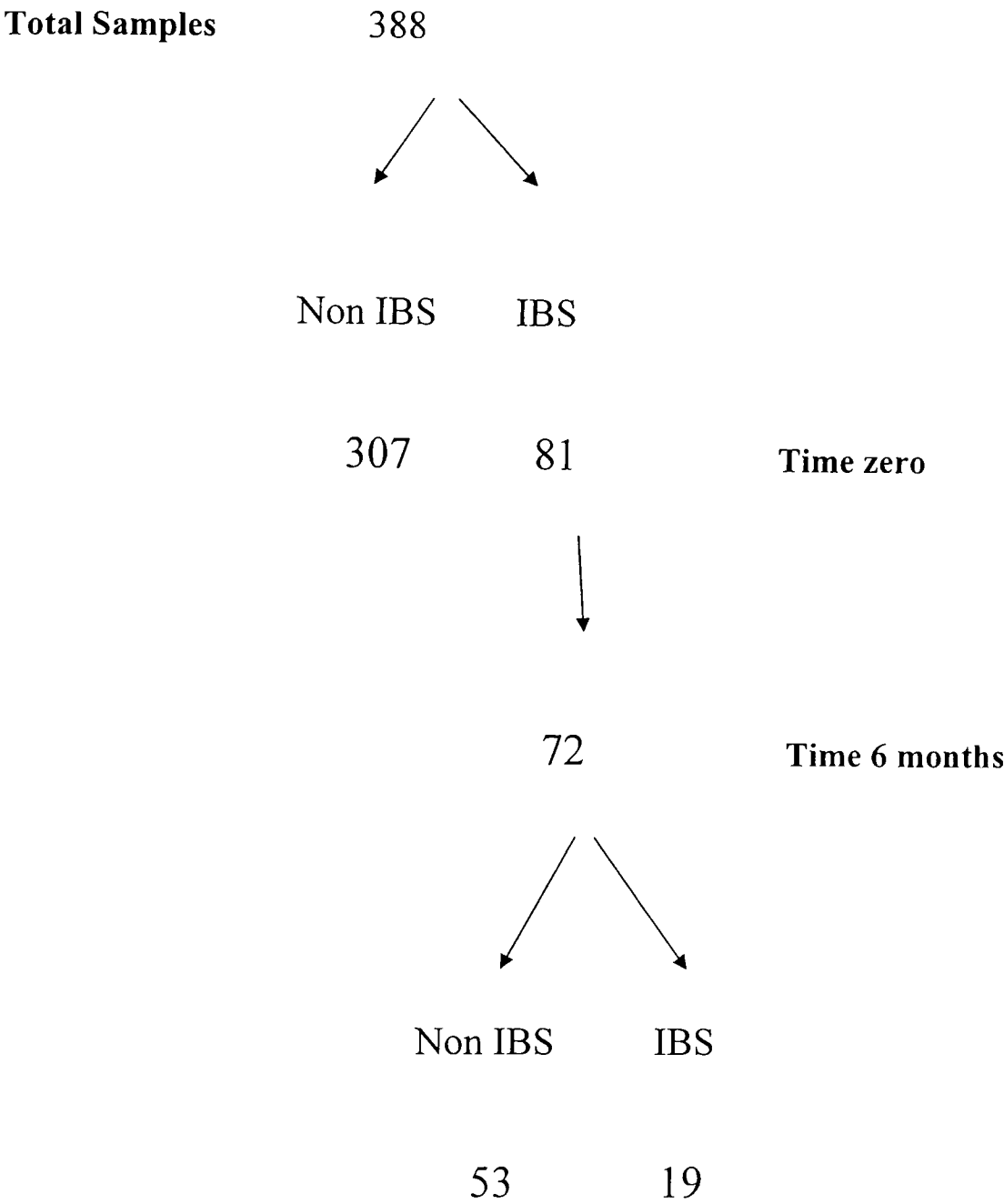


Fig 4.1 Breakdown of patient numbers and diagnosis from Giacometti *et al* (1999)

The choice of using the criterion of 5 organisms per field to identify positive patients is not justified. Microscopy is not so accurate and what if some people had 4 organisms per field, for example, should they be considered in this study or not? is this significantly different

from 5 or 6 ? It seems very arbitrary as a cut-off point and there is no justification given for its use- it is only random number.

Some people may have had more than 5 organisms per field on average but have been missed in diagnosis due to the sample of stool examined or it may be that *Blastocystis* was not the typical shape, for example only cysts or amoeboid stages. Both Udkow *et al.* (1993) and Zaki *et al.* (1991) reported that patients who do not show any symptoms may present with large numbers of *Blastocystis* cells in their faeces. This makes the choice of measuring cell density strange. In addition, stool culture was not used in this study despite showing more sensitivity than stool microscopy in a number of studies.

Tungtrongchitr *et al.* (2004) performed one study with a cohort of 59 IBS patients (27 male; 32 female) that were diagnosed by physical and biochemical laboratory examinations for the inclusion criteria of Roma Criteria II, and with 25 normal individuals used as control (Table 4.5). These individuals attended the Out-patient Department-General Practice sections of Siriraj Hospital and Ramathibodi Hospital, Bangkok. All patients provided one stool sample for analysis. Wet mount smears were prepared and evaluated under the microscope using saline and iodine solution for the presence of parasites. Trichrome, modified Trichrome and acid fast staining were also used. *Blastocystis* culture was carried out using monophasic medium.

The authors showed that there is no statistical difference between *Blastocystis* in IBS and control patients ( $P= 0.87$ ).

**Table 4.5** Prevalence of parasitic infections in irritable bowel syndrome (IBS) and control subjects

(From Tungtrongchitr *et al.* 2004)

Parasite	IBS (N=59)	Control (N=25)	P-value
<i>B.hominis</i> (vacuolated form)	13.6% (8/59)	12.0 % (3/25)	0.87
Non-pathogenic protozoa ( <i>Endolimax nana</i> cyst)	5.1 % (3/59)	-	-
<i>Strongyloides stercoralis</i> Rhabditiform larva	1.7 % (1/59)	-	-
Hookworm egg	-	8.0 % (2/25)	-
<i>Giardia lamblia</i> cyst	1.7 % (1/59)	-	-

The authors claimed that their results were the same as those of Giacometti *et al.* (1999) but in fact they were not similar. In Giacometti’s paper the difference was very significant when they compared prevalence of *Blastocystis* in two patients groups (P=0.006) which supported an association between *Blastocystis* and IBS. The result compared in Tungtrongchitr *et al.* (2004) with the Giacometti *et al.* (1999) paper is only the prevalence of < 5 organisms per field in patients without symptoms and patients with IBS. Also the authors reported that the culture method was found to be more sensitive than other methods without using any statistical analysis test.

In another study Hussain *et al.* (1997) measured the IgG antibodies levels in patients with IBS and *Blastocystis* at the Aga Khan Hospital (n=22) and the Jinnah Hospital (n=34) in Karachi during a six month period, also 36 healthy volunteers considered as endemic controls that did not give any history of IBS and were negative for all intestinal parasites including *Blastocystis* were recruited from the staff at the Aga Khan Hospital (Table 4.6). Patients were diagnosed with IBS on the basis of Roma II Criteria. Although stool microscopic examination

with saline and iodine was used to detect other intestinal parasites, the presence of *Blastocystis* in the stools was detected by culturing the organisms in Jones' medium.

The distribution of age and gender was similar in all groups. Patients with IBS showed increased levels of IgG antibodies irrespective of the presence or absence of *Blastocystis* in their stools. Hussain *et al.* (1997) also mentioned that in some patients the stool samples may have been negative for *Blastocystis* as they had received previous treatment with metronidazole.

**Table 4.6** Percentage seropositivity in patients with irritable bowel syndrome (IBS) (From Hussain *et al.* 1997)

Group	IgG	IgG1	IgG2	IgG3	IgG4
IBS (BH+) (n=23)	65	8.7	47.8	0	0
IBS (BH-) (n=32)	55	0	46.8	12.5	0
Control (n=36)	45	0	20	0	0

In this article four antibodies levels were measured: total IgG and IgG subclasses IgG1, IgG2 and IgG3. They observed a low level of IgG1 in IBS patients only when the stool was positive for *Blastocystis* (Table 4.7), whereas no IgG3 was seen in IBS patients with *Blastocystis*. However the level of IgG2 was similar in IBS patients with and without *Blastocystis*. Also the authors mentioned that patients with IBS showed increased levels of overall IgG antibodies irrespective of the presence or absence of *Blastocystis*.

In the paper title the authors state that IgG2 antibody level to *Blastocystis* increased significantly in patients with IBS but this is not what is stated in the result section: IgG2 antibody was similar in patients with or without *Blastocystis*. The link is between IBS and

control patients where the authors showed that IgG2 increased in IBS compared with healthy control group. I think the conclusion in the title is misleading.

In general there are some criticisms for each of these four papers.

For Yakoob's paper: the control group may not be a good control as it showed a higher percentage of diarrhoea than the IBS group. No description of the diarrhoea was given whether it was loose, watery, semi-soft or mucus diarrhoea. This may be significant.

For Giacometti's paper: many of the non-IBS patients as classified at 6 months follow-up were previously considered as IBS and had been used in comparisons between IBS and non-IBS at recruitment. Since IBS is a chronic disease this suggests a lot of the initial diagnosis may have been incorrect. Using the criterion of  $<$  or  $>$  5 organisms per field to identify infected and uninfected patients is not appropriate when investigating whether there is a link between *Blastocystis* and IBS. It is arbitrary.

For Hussain's paper: They detected increased level of IgG antibodies in presence or absence of *Blastocystis* organism in patients with IBS. The significance is unclear. Also they claimed that stool negativity in these patients may be due to previous treatment with metronidazole before presenting at the hospital. I believe this could bias the results by using patients who already received treatment.

For Tungtrongchitr's paper: the author collected only one stool sample from each patient and this could be a bias in the result, as other authors like Vennila et al. (1999) have mentioned that the shedding pattern of *Blastocystis* in infected persons is irregular.



The number of IBS samples is more than twice that of the control group and this could affect the result. The authors revealed that IBS patients who had *Blastocystis* were given metronidazole (1,200mg for 7 days), but they could not be fully cured using this standard treatment. It is possible that this resistance of *Blastocystis* could be linked to subtypes.

In summary, none of the four papers show any strong evidence of whether there is a link or not of *Blastocystis* to IBS. More research needs to be carried out to prove or disprove this link.

**Table 4.7** Different studies of IBS and control groups in several countries

Country	Reference	Method	Frequency of detection in IBS patients	Frequency of detection in control
Italy	Giacometti et al., 1999	Stool microscopy	19% (15/81)	8% (23/307)
Pakistan	Yakoob et al., 2010a	Stool culture	60% (95/158)	24% (38/157)
		Stool microscopy	55% (87/158)	20% (32/157)
Pakistan	Yakoob et al., 2010b	Stool culture	53% (90/171)	16% (25/159)
		Stool microscopy	48% (83/171)	17% (27/159)
Pakistan	Yakoob et al., 2004	Stool culture	46% (44/95)	7.3% (4/55)
		Stool microscopy	32% (30/95)	7.3% (4/55)
Turkey	Dogruman-Al et al., 2010	Stool microscopy	38% (8/21)	12% (5/43)
Mexico	Ramirez-Miranda et al., 2010	Stool microscopy	16% (18/115)	12% (25/209)
United Kingdom	Windsor et al., 2001	Stool microscopy	38% (>800 samples)	7% (64)
Thailand	Tungtrongchitr et al., 2004	Stool culture	14% (8/59)	12% (3/25)
		Stool microscopy	8.5% ( 5/59)	8% (2/25)
Thailand	Surangsrirat et al., 2010	Stool culture	17% (11/66)	10% (6/60)

Currently, few studies have been performed to investigate any association of *Blastocystis* subtypes and IBS. Two studies have investigated this link, one by Droguman-Al *et al.* (2009) and the other by Yakoob *et al.* (2010a). In Droguman-Al *et al.* (2009) the number of samples was only 21 (5 from IBS and 16 from control). Three subtypes were detected (ST1, ST2 and ST3, including mixed infections). ST3 was the most frequent in controls whereas ST2 was higher in IBS compared to controls and ST1 was detected only in controls. Two mixed

infections were detected in the control group. Due to the number of samples in Droguman-Al *et al.* (2009) the study of Yakoob *et al.* (2010a) is more useful as the number of samples is high (Table 4.7).

**Table 4.8** Distributions of *Blastocystis* subtypes from IBS and the unselected control group samples

Reference	Country	Patient group	Samples numbers	ST1	ST2	ST3	ST4	ST5	ST6	ST7	ST8	MIX ST	Unknown ST	Total
Yakoob et al., 2010a	Pakistan	IBS	123	75 (61 %)	6 (5 %)	23 (19 %)	6 (5 %)	3 (2.4 %)	3 (2.4 %)	5 (4 %)	–	–	2 (2 %)	179
		Unselected	56	12 (21.4 %)	4 (7 %)	26 (46.2 %)	2 (4 %)	4 (7 %)	3 (5.4 %)	5 (9 %)	–	–	–	
Present study	UK	IBS	116	13 (11 %)	8 (7 %)	46 (40 %)	44 (38 %)	–	1 (1 %)	1 (1 %)	3 (3 %)	–	–	225
		Unselected	109	19 (17 %)	11 (10 %)	49 (45 %)	25 (23 %)	2 (2 %)	–	2 (2 %)	1 (1 %)	–	–	

In my UK study eight subtypes were detected while in Yakoob *et al.* (2010a) only seven subtypes were identified, plus two unidentified samples. *Blastocystis* ST1 and ST3 were the major subtypes in Pakistan group whereas ST 3 and ST4 were in UK. In the Yakoob study, ST1, 2, 3, 4, 5, 6 and 7 infection were present in both IBS and control. In our study all eight subtypes were detected in both groups except ST5 in IBS and ST6 in the control group. ST3 showed a similar percentage in both countries and groups except in the IBS group from Pakistan where the prevalence was low (19%). The frequency of ST1 in IBS from Pakistan was very high (61%) and was five times higher compared to IBS in UK. The distribution of ST1 from both control groups and IBS samples from UK showed not much difference. ST4 was highest in the IBS group in UK (38%) while in IBS samples from Pakistan it showed a very low percentage (5%).

ST5, 6, 7 and 8 were detected as sporadic cases within both studies. ST8 was identified in UK with low percentage in both groups but none were detected from Pakistan. Worldwide only Scicluna *et al.* (2006) and Stensvold *et al.* (2009) have detected ST8 in humans. In ST2

there was not much variation between the two groups in both countries, while ST6 and ST7 showed a higher percentage in Pakistan compared to the UK.

Statistical analysis showed that there was no significant difference between *Blastocystis* subtypes and patient groups in UK ( $P=0.082$ ). In Pakistan, the  $P$  value is less than 0.001 which means that there is a highly significance difference between IBS and control group subtypes due to the high prevalence of ST1 in IBS (Table 4.9).

Both the control and IBS samples that we received came from different IBS clinics and non-IBS patient sources. We do not know whether some patients in the control group in fact have IBS. Similarly samples received from IBS clinics also could actually be from individual eventually diagnosed with other conditions. For this reasons our study groups are not very well defined and this could affect our results. For example if ST4 in the control group was lower after excluding patients with symptoms consistent with IBS then this may alter the significance of the  $P$  value. To design a better study we need to identify patients with a definite diagnosis of IBS for the IBS group and if possible only asymptomatic individuals in the control group. The IBS patients will be easier to identify than a source of asymptomatic individuals infected with *Blastocystis* as only people with illness usually submit stool samples for analysis.

There is an indication that ST4 is associated with IBS in the UK although it is not statistically significant. However, this association is not seen in Pakistan where ST1 is more common in IBS patients and ST3 is less common. If both results are real it suggest that other factors are influencing the association of subtype with IBS, perhaps strains within STs.

## The objective

### To investigate *Blastocystis* subtypes in IBS patients

Subtypes in IBS patients were investigated but no significant association was found.

However, these are problems with the samples used.

In order to investigate any link between *Blastocystis* STs and IBS we need to get faecal samples from both IBS patients and a control group. In order to do this we have to get true IBS patients. This can be done through contact with IBS clinics in order to have complete medical information about each IBS patient and the final diagnosis in order to ensure that they are real IBS patients. The same thing applies to the control group. It is important to be sure that they do not meet the Roma criteria and so do not have IBS. It is also important that they match the IBS group as closely as possible except for the diagnosis. This sort of study was not possible in my case due to various reasons, including time and labour but also because IBS and control samples were received through the Diagnostic Laboratory at LSHTM and we do not get access to important information about each patient like clinical history, history of contact with animals, their occupation and where they are living.

Once we have samples with all this information, it will be possible to investigate *Blastocystis* STs in each group, and see whether there is any significant difference between the ST distributions. With the sample group that was available there are too many factors that are unknown.

## **Chapter 5**

# ***Blastocystis* subtypes in domestic and zoo animals**

## 5.1 Introduction

*Blastocystis* infects humans, amphibians, reptiles, cockroaches and a wide range of mammals and birds (Tan, 2004, Abe, 2004; Yoshikawa *et al.*, 2007; Yoshikawa *et al.*, 2004; Teow *et al* 1991). *Blastocystis* has been reported in many parasite surveys of animals in zoological gardens, especially in non-human primate species (Abe, 2002), while studies of *Blastocystis* in domestic animals have also revealed high frequency of occurrence in cattle, pigs and birds (Yamada *et al.*, 1987, Abe *et al.*, 2002).

Subtype studies of *Blastocystis* infection in humans have shown that some *Blastocystis* isolates from mammals and birds have the same subtypes seen in humans and so seem to have zoonotic potential (Clark 1997; Salim *et al.*, 1999). Therefore it has been proposed by many authors that human infections may result from zoonotic transmission of the parasite, but this remains to be established.

At the beginning of this study nine STs had been described in mammals and birds (Fig 1.5). In 2009, ST10 was reported in cattle and non-human primates (Stensvold *et al.*, 2009) and in 2010 STs 11-13 were reported by Parkar *et al.* (2010) in a variety of zoo animals. The aim of this study was to determine the STs of *Blastocystis* in zoo and domestic animals from two different countries, the UK and Libya, and to investigate the degree of host specificity among these STs. In order to investigate the hypothesis (animals are the source of *Blastocystis* in humans) we need to know the host range and STs of *Blastocystis* in a variety of animals

In Libya there is frequent human exposure to animals but no information whereas in the UK there is a limited human exposure, but only data on zookeepers who works with monkeys. In the UK, in general people may get exposure or contact with animals like dogs, cats, ducks or

squirrels but not to monkey, cow, sheep or goat. There are limited people who have frequent exposure to animals in UK like zookeepers. For this study I did have samples from zookeepers, however the numbers are very small and this is a limitation to my study. In Libya, people get exposure to different animals like sheep, cow, goat, camel, chicken and even rodent. We do have samples from these animals but from animal handlers we tried hard to get samples from them but they refused. I consider this as another limitation to my work and I think data questionnaire for Libyan human samples from clinics may help to gather some information about animal exposure and *Blastocystis* infection in Libya in future. Even so, the information on *Blastocystis* subtypes in different animal hosts is an important part of that future study and the results will be reported here.

## **5.2 Materials and methods**

### **5.2.1 Source of specimens UK**

#### **5.2.1.1 UK animal samples**

105 faecal samples from non-human primates and two mouse deer were received for routine parasitological investigation by the Diagnostic Parasitology Laboratory in the London School of Hygiene and Tropical Medicine from various animal facilities and collections (Table 5.1). Samples were processed as indicated in chapter 2. We were dependent on zoos submitting samples, so the number and range of samples was limited. Most samples were from individual monkeys and received over a period of several years but some were samples from groups of animals living in the same enclosure so the individual animal providing the sample could not be identified. Most hosts were represented by only a small number of samples and so the range of subtypes found in those species is likely to be incomplete.

**Table 5.1** List of animal hosts and zoo location in UK

Host	Animal zoo location								Total
	Monkey World	Paignton	Twycross	Monkey Sanctuary	Colchester	Chessington	Woburn	Unknown	
Chimpanzee	11	1	—	—	—	—	—	2	14
Gorilla	—	3	—	—	—	2	—	—	5
Woolly Monkey	14	—	5	1	—	—	—	—	20
Macaque	2	4	—	2	—	—	—	—	8
Orangutan	7	4	—	—	—	—	—	—	11
Gibbon	6	4	—	—	1	—	—	—	11
Diana Monkey	—	4	—	—	—	—	—	—	4
Baboon	—	2	—	—	—	—	—	—	2
Lemur	—	2	—	—	—	—	—	—	2
Allen Swamp Monkey	1	2	—	—	—	—	—	—	3
Colobus	—	1	—	—	—	—	2	—	3
Patas Monkey	—	—	—	2	2	—	—	—	4
Siamang	3	—	—	—	—	—	—	—	3
Langur	—	—	3	—	—	—	—	—	3
Marmoset	1	—	—	—	—	—	—	—	1
Mandrill	—	3	—	—	—	—	—	—	3
Black Howler Monkey	—	—	2	—	—	—	—	—	2
Unknown monkeys	1	1	—	—	—	—	—	2	4
Mouse deer	—	2	—	—	—	—	—	—	2
Total	46	33	10	5	3	2	2	4	105

**5.2.1.2 UK human samples**

34 faecal samples were submitted by zoo keepers who work closely with non-human primates in zoos in the UK (Table 5.7). These samples were submitted for routine ova and parasite screening to the Diagnostic Parasitology Laboratory.

**5.2.1.3 Libyan animal samples**

A total of 309 faecal samples were collected from various animal hosts in Libya during July 2008- August 2010 to determine the range of *Blastocystis* parasites present in this country. Samples were collected from different animals from different parts of Libya including camel, cattle, goat, donkey, horse, gazelle, Barbary sheep and gundi. In the city of Sebha, 248 faecal samples were collected and analysed. One faecal sample per animal was taken at each



collection time from each farm. A total of 33 faecal samples from Zawia were investigated and 13 and 15 samples were collected from Garaboli and Zahira respectively.

Camel samples were collected from six farms in Sebha. The distance between farms varied between 5 and 40 KM and the number of camels in each farm varied from 30 to 1000 head.

Goat and cattle samples were collected from three farms in Sebha plus 13 samples from cattle were collected from Garaboli as well. Samples from gundi, gazelle and Barbary sheep were collected at a tourist camp in Sebha

Two donkey samples were collected from Sebha and the rest from Zahra; horse samples were collected from Zahra only.

For all animals, faecal samples were collected directly after defecation by the animal so collection was dependent on observing defecation. This was so that the faeces was as fresh as possible. For the gundi, the people who look after them helped me to collect the samples by holding the animals until the samples could be collected. Gloves were worn at all times while collecting samples from the animals.

Obtaining a stool sample from most animals was easy and it was not necessary to catch the animal, but it could be picked up where it lay. Most animals defecate after meals, so by watching the animal after feeding the animal soon passed faeces and I collected it. To prevent contamination I could not just scoop it up off the ground but had to be very careful not to take any soil or plant matter. I used a very clean disposable implement, such as a tongue depressor or cotton swab, to scoop a faecal sample into a container, and I avoided cross- contamination by using gloves and washing my hands after collection.

After samples had been collected from an animal I labelled the faecal container according to animal type and gave it a serial number, location of the farm, date and time of collection. To investigate the presence of *Blastocystis*, in the laboratory all samples were examined using direct smear microscopy and culture was performed using modified Robinson’s culture. This occurred as soon as possible after collection, within 6 hours.

In Sebha, samples were collected from several animal hosts but the most samples were collected from camels. Six farms in Sebha were studied for *Blastocystis*.

**Table 5.2** Animal host samples and farm locations in Libya

Location	Farm	Animal host									Total
		camel	cattle	goat	Barbary sheep	gazelle	gundi	donkey	horse	chicken	
Sebha	1	30									30
	2	19									19
	3	59									59
	4	34									34
	5	15									15
	6	6									6
	7		6	10							16
	8		7								7
	9		8					2			10
	10		2								2
	11			12				1			13
	12			11							11
	13			5							5
	14				5	9	4				18
	15									3	3
Garaboli	16		13								13
Zahra	17							13			13
	18								2		2
Zawia	19	33									33
Total		196	36	38	5	9	4	16	2	3	309

An additional 33 samples were collected from one camel farm from Zawia. Goats provided the second largest number of samples and all were collected from Sebha.

Cattle samples were collected from both Sebha and Garaboli. Samples were handled as described in chapter 2.

### **5.2.2 Sample analysis**

*Blastocystis* isolation, DNA extraction, PCR and Sequencing were performed as in chapter 2, sections 2.1-2.6. The only exception is 13 samples from cow where DNA was extracted directly from faeces (Table 5.4).

## **5.3 Results**

414 faecal samples were examined from animal hosts in both the UK and Libya. In addition 34 faecal samples from zoo keepers from the UK were analysed. 29 primate keepers from Monkey World were compared with the non-human primates from the same zoo to investigate whether there was any evidence of transmission of *Blastocystis* between them.

13 STs were recognized by barcoding including four new subtypes (ST14, ST15, ST16 and ST17) that are described below.

### **5.3.1 Libyan animal samples**

From the total number of 309 Libyan animal samples examined using culture for *Blastocystis*, 141 (47.6 %) gave positive results. The incidence of *Blastocystis* in these animals is illustrated in Table 5.3.

**Table 5.3** The incidence of infected animals with *Blastocystis* in Libya.

Host	Positive by culture	Negative by culture	Total
Camel	111 (57%)	85 (43%)	196
Cow	2 (9%)	21 (91%)	23
Goat	17 (45%)	21 (55%)	38
Gundi	4 (100%)	—	4
Gazelle	4 (44%)	5 (56%)	9
Barbary sheep	2 (40%)	3 (60%)	5
Donkey	—	16 (100%)	16
Horse	—	2 (100%)	2
Chicken	1 (33.3%)	2 (66.7%)	3
Total	141 (47.6%)	155 (52.4%)	296

141 samples were positive by culture, and PCR were performed on all these samples but only 70 gave positive PCR (Table 5.4). In addition 13 samples extracted directly from cow faeces were positive. This percentage could be due to different reasons; either presence of inhibitors within samples, low DNA degradation, or during the preparation of the samples the DNA may have been lost. It is also possible that the organisms in the negative PCRs were divergent in the region of the BhrDR primer and so the amplification did not work, however the primer is conserved in all known *Blastocystis* to date.

**Table 5.4** PCR results of animals infected with *Blastocystis* in Libya.

Host	Positive by PCR	Negative by PCR	Total
Camel	47 (42.3%)	64 (57.7%)	111
Cow	*15 (100%)	—	15
Goat	4 (24%)	13 (76%)	17
Gundi	1 (25%)	3 (75%)	4
Gazelle	1 (25%)	3 (75%)	4
Barbary sheep	1 (50%)	1 (50%)	2
Chicken	1(100%)	—	1
Total	70 (45.5%)	84 (54.5%)	154

\*Includes 13 samples of *Blastocystis* DNA extracted directly from cow faeces.

There were nine subtypes detected in Libyan animals with four considered to be new subtypes, collected from camel (*Camelus dromedarius*), cow and gundi (*Ctenodactylus gundi*) (Table 5.5). Identification of the four new subtypes was based on sequence of the bar-coding region compared with sequence of other subtypes from GenBank. They showed more than 3% nucleotide divergence from the known subtypes. Comparison of the whole gene sequence of SSU rDNA with complete sequence of other subtypes also confirmed that they were distinct.

**Table 5.5** Distribution of *Blastocystis* subtypes in Libyan mammals and birds

Host subtype	ST1	ST2	ST3	ST4	ST5	ST6	ST7	ST8	ST9	ST10	ST11	ST12	ST13	ST14	ST15	ST16	ST17	Mix ST	Total
Camel	5(11%)	—	5(11%)	—	20(43%)	—	—	—	—	6(13%)	—	—	—	—	2(4%)	1(2%)	1(2%)	7(15%)	47
Cow	—	—	—	—	2(13%)	—	—	—	—	6(40%)	—	—	—	—	—	2(13%)	—	5(33%)	15
Goat	—	—	1(25%)	—	—	—	1(25%)	—	—	1(25%)	—	—	—	—	—	—	—	1(25%)	4
Gundi	—	—	—	—	—	—	—	—	—	—	—	—	—	1(100%)	—	—	—	—	1
Barbary sheep	—	—	—	—	—	—	—	—	—	1(100%)	—	—	—	—	—	—	—	—	1
Gazelle	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1(100%)	1
Chicken	—	—	—	—	—	—	1(100%)	—	—	—	—	—	—	—	—	—	—	—	1
Total	5(7%)	—	6(9%)	—	22(31%)	—	2(3%)	—	—	14(20%)	—	—	—	1(1.4%)	2(2.8%)	3(4.3%)	1(1.4%)	14(20%)	70

Overall, ST5 shows the highest frequency (31%) with ST10 is in the second position with 20%. Out of all the animals, camels have more STs but also represent the most samples, The number of STs in cow and goat were low possibly because the numbers of samples were low as well. ST5 is more common in camels (41%) than other animals, while ST1, ST3 and ST10 all occur in camel with similar prevalence (11-13%). Both camel and cow showed more mixed infections than other animals. Chicken, barbary sheep (*Ammotragus lervia*) and gundi demonstrated only one subtype each, carrying ST7, ST10 and ST14 respectively. In the gazelle (*Gazella leptoceros*) no subtype could be assigned because it showed only a mixed infection.

ST10 was the subtype with the widest host range and was detected in four animals, camel, cow, goat and barbary sheep, whereas ST3, ST5, ST7 and ST16 were each detected from two different hosts. Some subtypes were detected only in one specific animal: ST1, ST15 and ST17 were found only in camels while ST14 was detected only in gundi (Table 5.5).

In cows, three subtypes were detected (ST5, ST10, and ST16) and among these subtypes ST10 had the highest frequency (40%). The two ST5 samples from cow show some divergence from other ST5 sequences. Complete gene sequences will be necessary to decide whether these need to be assigned to a new subtype. In goats three subtypes were seen as well, ST3, ST7 and ST10, with equal frequency. Donkeys and horses did show any *Blastocystis* in their faeces, either by direct smear or by culture.

### **5.3.2 UK animal samples**

In the UK, 105 samples were collected from non-human primates (NHP) and two samples from mouse deer. Nine subtypes were identified, with one new subtype (ST17) detected from various hosts including chimpanzee, gibbon, lemur and an unidentified monkey. This subtype was also detected in a Libyan camel (see above). A few samples showed mixed subtype infections (Table 5.6).

**Table 5.6** *Blastocystis* subtype distribution identified in non-human primates and other mammals from the UK (n=105)

Host	ST1	ST2	ST3	ST4	ST5	ST6	ST7	ST8	ST9	ST10	ST11	ST12	ST13	ST14	ST15	ST16	ST17	MIX ST/Bad seq	PCR ( - )	Total
Chimpanzee	—	4	3	—	3	—	—	1	—	—	—	—	—	—	—	—	1	1	1	14
Gorilla	—	1	1	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	2	5
Woolly monkey	—	1	3	—	—	—	—	14	—	—	—	—	—	—	—	—	—	—	2	20
Macaque	1	—	7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	8
Orangutan	2	4	2	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	1	11
Gibbon	—	2	1	—	1	—	—	3	—	—	—	—	—	—	—	—	1	2	1	11
Diana Monkey	2	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4
Baboon	—	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2
Lemur	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	1	2
Allen Swamp Monkey	—	—	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3
Colobus	—	—	2	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	3
Patas Monkey	1	—	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4
Siamang	2	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	3
Langur	—	—	2	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	3
Marmoset	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1
Mandrill	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3
Black Howler Monkey	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—	—	2
Unknown monkeys	1	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1	—	4
Mouse deer	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	2
Total	12	12	33	—	7	—	1	22	—	—	—	—	2	—	—	—	4	4	8	105

ST3 is the dominant subtype in NHPs with a high frequency seen among macaques in particular (Table 5.6). This is also the most common ST in humans. However, ST8 is the second most common in NHPs and was most common in woolly monkeys. ST8 is very rare in humans, except for zoo keepers (Scicluna *et al.*, 2006).

In Table 5.7 we compare STs in non-human primates with STs in zoo keepers from the same zoo. ST8 was the highest ST in NHP from monkey world and found in more than 13 % of zoo keepers. ST3 is the most common ST in zoo keepers. ST4 was not detected from any NHP but shows 10.3 % in zoo keepers. Most woolly monkey samples have ST8 but we do not know how many zookeepers with ST8 were working with woolly monkeys because they rotate among primate groups..

**Table 5.7** *Blastocystis* subtype distribution in zoo keepers and non-human primates in Monkey world zoo in UK.

Host	Subtype								Total
	ST1	ST2	ST3	ST4	ST5	ST6	ST7	ST8	
Non-human primates	4 (10%)	10 (25%)	8 (20%)	—	2 (5%)	—	—	16 (40%)	40
Zoo keepers*	7 (24.1%)	5 (17.2%)	9 (31%)	3 (10.3%)	—	—	1 (3.4%)	4 (13.8%)	29
Total	11 (15.7%)	15 (21.4%)	17 (24.3%)	3 (4.3%)	2 (2.9%)	—	1 (1.4%)	20 (28.6%)	70

\*In addition, there were three with negative PCR and one with bad sequence

ST6 was not detected in any NHP host whereas ST7 was identified only in one sample from a colobus monkey (Table 5.6) and one zookeeper (Table 5.7)

Mouse deer can be considered a new host for *Blastocystis* and was infected with ST13 which was recently reported in a quokka by Parkar *et al.* (2010). This means that ST13 is not confined to marsupials. No infections with ST6, ST9, ST11 or ST12 were detected in this study, in either UK or Libyan animal samples.

**5.3.3     Phylogenetic analysis**

There were four new subtypes detected in this study. However this conclusion was based initially only on the barcode region. To confirm this result and to investigate how the new STs are related to the others, the complete gene sequence was obtained.

**5.3.4     PCR amplification**

The amplification of the SSU rRNA coding regions of 5 *Blastocystis* isolates from gundi, camels, cows, monkeys and mouse deer analyzed in this study was carried out as described in

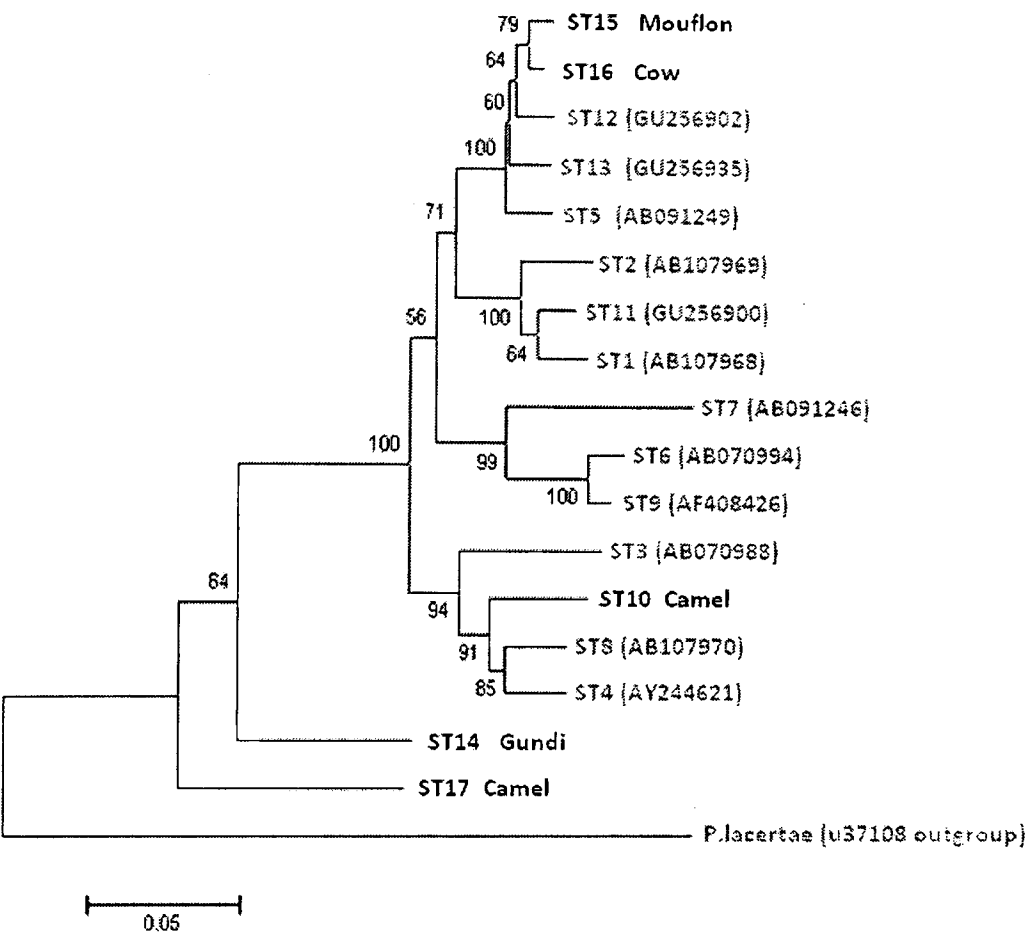


Chapter 2 using primers RD5 and RD3 to produce a DNA fragment of the expected size (~ 1.8 kb in length) as determined by gel electrophoresis. ST10 was originally identified based on small regions only (Stensvold *et al.*, 2009) and so a gene for this subtype was sequenced completely in this study in addition to the new subtypes. Sequences obtained from the five *Blastocystis* isolates in the present study were aligned with the SSU rRNA sequences of 12 *Blastocystis* sequences collected from GenBank representing different subtypes for phylogenetic analysis (Table 5.8). These 12 isolates have been sequenced previously along the entire or partial (Parkar *et al.*, 2010) length of the SSU rDNA. Since the nucleotide sequences of SSU rDNA for ST11, ST12 and ST13 were incomplete, as reported by Parkar *et al.* (2010), we decided to use the same region (1100 bp) from each ST to construct the phylogenetic tree (Fig 5.1). Two isolates from camel, one from Sebha and the other from Zawia, showed similar sequences to an unpublished one found in a mouflon in the barcoding region and since the whole gene of SSU rRNA of the mouflon sample already been sequenced (Taner-Mulla, 2010) we decided to use that sequence to construct the phylogenetic tree instead of sequencing ST15 again.

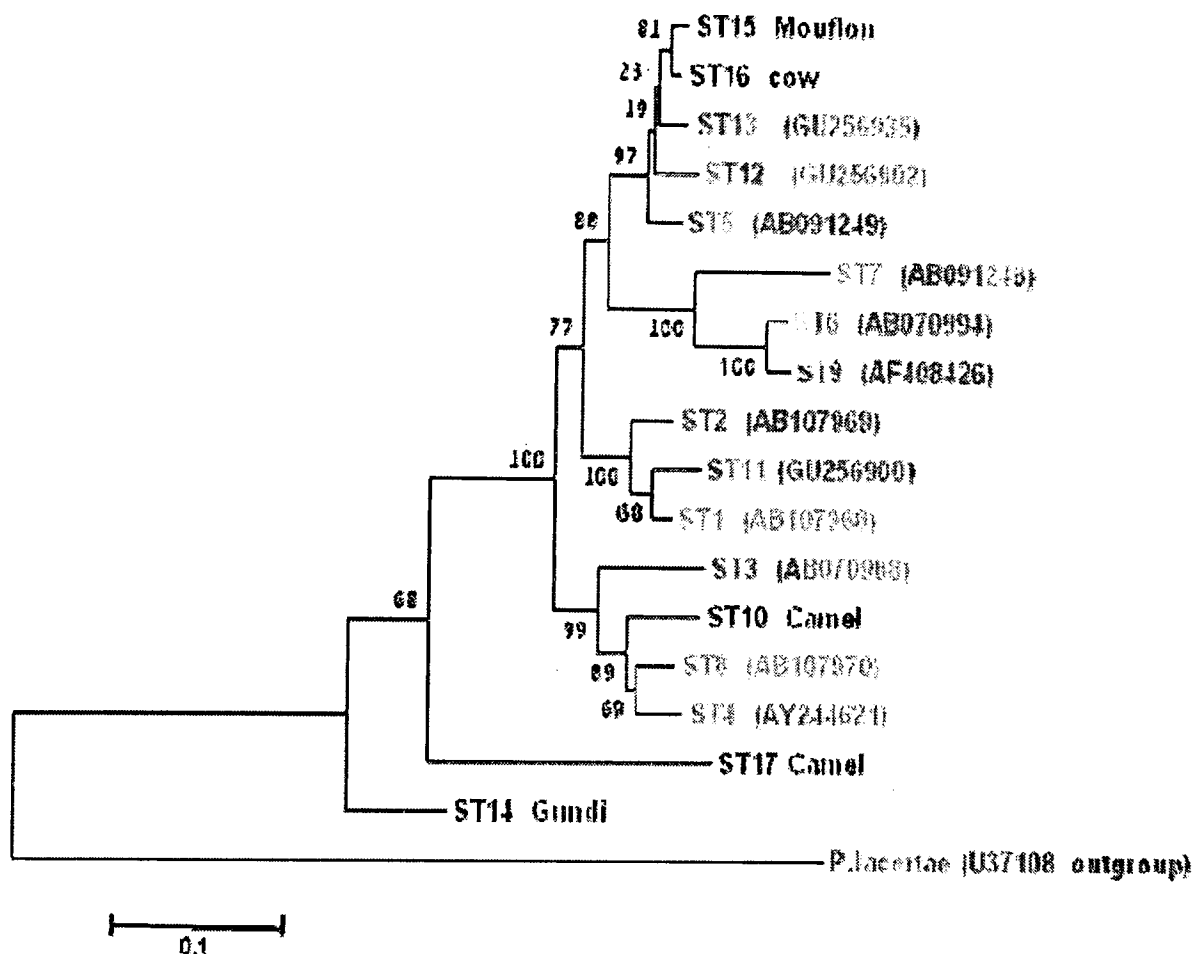
**Table 5.8** Reference sequences for each *Blastocystis* subtype included in this work with their GenBank accession number, host, location and reference

ST	Host	Location	GenBank	Reference
ST-1	Primate	Japan	AB107968	Abe (2004)
ST-2	Primate	Japan	AB107969	Abe (2004)
ST-3	Human	Japan	AB070988	Arisue et al. (2003)
ST-4	Human	Japan	AY24462	Yoshikawa et al. (2003)
ST-5	Pig	Japan	AB091249	Arisue et al. (2003)
ST-6	Chicken	Japan	AB070994	Arisue et al. (2003)
ST-7	Chicken	Japan	AB091246	Arisue et al. (2003)
ST-8	Primate	Japan	AB107970	Abe (2004)
ST-9	Human	Japan	AF408426	Yoshikawa et al. (2004)
ST-11	Elephant	Australia	GU256900	Parkar et al. (2010)
ST-12	Giraffe	Australia	GU256902	Parkar et al. (2010)
ST-13	Quokka	Australia	GU256935	Parkar et al. (2010)

The flagellate *Proteromonas lacertae* was used as the outgroup based on earlier phylogenetic studies (Noël *et al.*, 2003). The rooted neighbour-joining tree identified 17 lineages (Fig 5.1). The relationships among the STs already known those reported in the previous studies. Based on degree of divergence, there is a strong case for placing all our new sequences as separate novel lineages or STs, and we allocated them to ST14, ST15, ST16 and ST17 (Fig 5.1).



(A).



(B).

Fig. 5.1 Phylogenetic tree of the new *Blastocystis* sequences and reference SSU rRNA gene sequences from GenBank. This is based on 1100 aligned base pairs of the SSUrDNA and was rooted on *Proteromonas lacertae*. The values on the nodes are bootstrap support values based on 1000 replicates for (A) Neighbour-joining (NJ) and 100 replicates for (B) Maximum likelihood (ML).

To illustrate the differences in nucleotides between 17 ST of *Blastocystis* based on the SSU rRNA gene, the sequence of our four new STs was aligned with the other 13 known *Blastocystis* STs sequences (Fig 5.2)



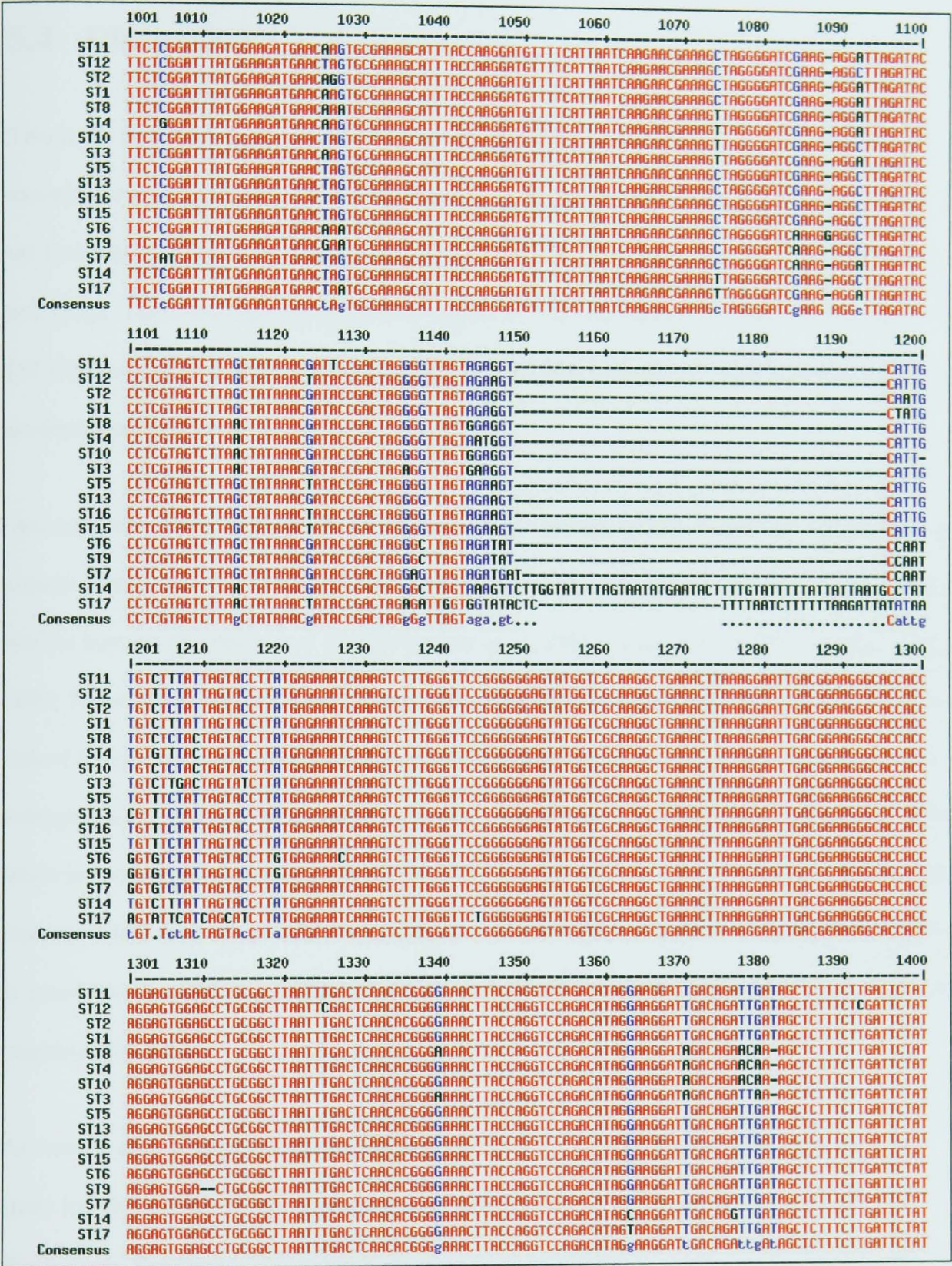


Fig 5.2 Alignment of nucleotides sequence from 17 STs.

The region showed corresponds to 1101-1500 of the small subunit (SSU) rRNA gene of *Blastocystis* sp. subtypes.

## 5.4 Discussion

This is the first study to report *Blastocystis* isolated from Barbary sheep, gazelle, gundi and mouse deer which therefore should be considered as new hosts for this parasite. Also this is the first report of four new *Blastocystis* subtypes from various animal hosts in both the UK and Libya. These are from gundi (ST14), camel (ST15), cow and camel (ST16) and finally (ST17) from camel as well as from non-human primates (chimpanzee, gibbon, lemur and an unidentified monkey).

Animals have been proposed to be a reservoir for human infection with *Blastocystis* since certain subtypes of this organism have been discovered in an extensive range of animals as well as humans (Yoshikawa *et al.*, 2004a; Abe *et al.*, 2003a; Arisue *et al.*, 2003; Parkar *et al.*, 2007; Stensvold *et al.*, 2009) and people with close animal contact were found to be at higher risk of *Blastocystis* infection (Salim *et al.*, 1999). In this study, ST8 appears to be a zoonotic subtype, being found in non-human primates as one of the most common subtypes (40%) while in zoo keepers it showed a prevalence of 13.8% but in UK human random population it represents less than 1% of infections (Table 3.1). Although the number of zookeeper samples is small, the prevalence of ST8 in zookeepers (4/29) was much higher than in the general population (4/225).

As was the case in earlier studies (Abe *et al.*, 2002; Perez Cordon *et al.*, 2008), the present study has shown that many primates in zoos and many domestic animals are infected with *Blastocystis*. Relatively few studies have screened non-primate zoo animals for *Blastocystis* (Abe *et al.*, 2002, Lim *et al.*, 2008), but the recent paper by Parkar *et al.* (2010) suggests that additional host-specific subtypes exist.



In Libya, even though ST5 and ST10 were not found in humans, they were the most dominant subtypes in animals (50 % of samples) especially in camel and cow. This means that either those hosts do not contribute to human infections or these subtypes do not usually infect humans. Different authors have found ST5 in cattle and pigs (Abe *et al.*, 2003; Arisue *et al.*, 2003; Yan *et al.*, 2007; Yoshikawa *et al.*, 2004a; Stensvold *et al.*, 2009), while a few cases were detected in primates as in our study (Table 5.6). In Libya, none of the animal hosts carried ST2 (Table 5.5) but in humans this subtype was found in 8 % of infections (Table 3.1). Therefore this ST seems unlikely to be of zoonotic origin in Libya. ST10 was not detected in human samples either in our study or in any previous study (Table 6 chapter 3). However, it was detected in the most mammal hosts in Libya in this study (Table 5.5). Stensvold *et al.* (2009) detected ST10 in cattle and nonhuman primates while Santin *et al.* (2011) also detected ST10 in cattle. For the time being it appears that cattle are the most common host to carry ST10. In addition detecting ST10 in Libyan, European and American mammals means that there is no geographical restriction to the distribution of this subtype.

ST1 and ST3 are the most dominant subtypes in Libyan humans but are relatively rare in Libyan animals with 7 % and 9 % of samples respectively, mostly in camels. As people come in contact with camels on farms, animal markets or camel breeding places, it is possible that there is transmission of the parasite between the two hosts. As we do not know much information about the origin of the human Libyan samples, it might be that some infected people have been exposed to camels. It seems unlikely that camels would be the only source of infection, however.

According to previous studies, ST6 and ST7 are found only in birds and sporadic cases of human infections. In this study ST6 was not detected in any samples in either the UK or Libya, whereas ST7 was identified in goat, chicken (Table 5.5) and in humans as well (Table

3.1). Therefore, this result suggests that human isolates of ST7 could be of zoonotic origin and may show evidence of animal-to-human transmission. However, there is no proof of this.

Table 5.9 shows more information on the genetic diversity of *Blastocystis* in camels in Libya. Out of six camel farms, there were seven *Blastocystis* subtypes detected. However, ST10 and ST17 were detected on one farm only whereas ST 1, ST3, ST5 and ST15 were found on more than one farm. ST5 had the highest frequency and was detected in most of the farms.

Significant differences in ST distribution exist between camel farms even in the same region suggesting there is limited cross-infection occurring between farms.

During my work four new subtypes were discovered and these subtypes were proposed based on comparing the sequence of the whole SSU rRNA gene to other subtypes. Some subtypes, like ST14 which was detected in a gundi, was very diverged compared with other subtypes and were clearly new. ST17 which was found in two hosts, monkey and camel also showed large differences from other subtypes.

In contrast, some new STs were close to previously designed subtypes. Initially there were called ST5 variants. Whole SSU rRNA gene sequencing was carried out and a minimum divergence of 3% was used to distinguish between the potential new subtype and existing subtypes. In addition, I required that the 'new' subtype was separated from existing subtypes in phylogenetic trees with at least 99% bootstrap support. I recognise that future sampling may show that some of these new subtypes are not supported. As we get more sequences then new STs will either combine together with other STs to form one group or they will continue to be separate and confirmed as new.



The new *Blastocystis* ST17 that was detected on farm 2 is the same subtype as found in chimpanzee, gibbon, lemur and an unidentified primate in UK. Additionally, ST16 was found in two different hosts (two cows and one camel) (Table 5.5) that had been collected from different areas in Libya (Sebha, Garaboli and Zawia) (Table 5.2). These findings mean that there is no specific host or geographical distribution for these *Blastocystis* subtypes.

**Table 5.9** *Blastocystis* subtypes distribution in camels found in Libya

Subtype	ST1	ST2	ST3	ST4	ST5	ST6	ST7	ST8	ST9	ST10	ST11	ST12	ST13	ST14	ST15	ST16	ST17	Mix ST	Total
Farm 1 Sebha	—	—	1 (5%)	—	15(75%)	—	—	—	—	—	—	—	—	—	1 (5%)	—	—	3 (15%)	20
Farm 2 Sebha	—	—	—	—	—	—	—	—	—	6 (60%)	—	—	—	—	1 (10%)	—	1 (10%)	2 (20%)	10
Farm 3 Sebha	2 (50%)	—	2 (50%)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4
Farm 4 Sebha	1 (16.7%)	—	2 (33.3%)	—	1 (16.7%)	—	—	—	—	—	—	—	—	—	—	—	—	2 (33.3%)	6
Farm 5 Sebha	—	—	—	—	1 (100%)	—	—	—	—	—	—	—	—	—	—	—	—	—	1
Farm 7 Zawia	2 (33.3%)	—	—	—	3 (50%)	—	—	—	—	—	—	—	—	—	—	1 (16.7)	—	—	6
Total	5 (10.6%)	—	5 (10.6%)	—	20 (42.6%)	—	—	—	—	6 (12.8%)	—	—	—	—	2 (4.2%)	1 (2.1%)	1 (2.1%)	7 (15%)	47

Table 5.10 displays the STs of *Blastocystis* infecting 566 animals including the data from analysis of 168 isolates in the present study and identifiable ST data from all previously published studies.

It is clear from Tables 5.5 and 5.6 that a number of animals in both the UK and Libya showed mixed subtype infections while in humans there were no mixed infections detected. In the UK the frequency of mixed infection in primates was 3.8% whereas in Libya it reaches up to 20% of mammals. In previous studies of human populations overall mixed infection prevalence was only 3.3 % % (Table 3.2). Mixed infection in Libyan animals is higher compared with other groups and this could be due to animal husbandry practice in Libya where cow, camel, goat and sheep are often found in mixed groups feed is provided on ground where all the animals gather and eat in same place. The animals also defecate in the same place which gives more chance for animals to get infected with more than one ST.

In the UK, human and animal hosts shared the same range of STs except for ST13 (mouse deer) and ST 17 (non-human primates). Hence the genetic diversity of *Blastocystis* in both groups is not very different. In contrast, in Libya animals carried nine subtypes (Table 5.5) compared with only four subtypes in humans (Table 3.1). For example, both ST5 and ST10 are common in animals but ST5 in human is a rare and ST10 is not found at all. In addition ST8 is rare in general population but more common in people who worked with non-human primate. It is possible that if we were to examine people who are in close contact with animals the range of subtypes in humans would increase, but at present it appears that the range of subtypes in animals (16) is much greater than in humans (9) and that some host specificity exists.

Several *Blastocystis* STs reveal broad host ranges, although they may not all be of zoonotic significance. There are significant differences in prevalence among subtypes between hosts and between the UK and Libya. It is not yet clear how much of the variation is due to the surveyed hosts and how much to geographic origin. Wider sampling of cattle and other Artiodactyls in the UK would help to answer some of these questions.

**Table 5.10** *Blastocystis* subtype classification in non-human primates, other mammals and birds (n=566) (continued on next page)

Host	Blastocystis sp. Subtype (ST)																		Reference
	ST 1	ST 2	ST 3	ST 4	ST 5	ST 6	ST 7	ST 8	ST 9	ST 10	ST 11	ST 12	ST 13	ST 14	ST 15	ST 16	ST 17	Mix ST/ Bad seq	
Chimpanzee	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Abe et al.(2003b), Abe (2004)
	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al.(2004a)
	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	Stensvold et al.(2009)
	—	4	3	—	3	—	—	1	—	—	—	—	—	—	—	—	1	1	Present study
Gorilla	—	1	1	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	Present study
	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Santin et al.(2011)
Orang-utan	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Abe et al.(2003b), Abe (2004)
	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Parkar et al.(2007)
	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Parkar et al.(2010)
	1	1	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al.(2004a)
	2	4	1	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	Present study
Gibbons	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Abe et al.(2003b), Abe (2004)
	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Parkar et al.(2007)
	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	Stensvold et al.(2009)
	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Parkar et al.(2010)
	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al.(2004a)
	—	2	1	—	1	—	—	3	—	—	—	—	—	—	—	—	1	2	Present study
Baboon	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Parkar et al.(2007)
	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Parkar et al.(2010)
	—	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Present study
Mandrill	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Abe et al.(2003b), Abe (2004)
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al.(2004a)
	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Present study
	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Santin et al.(2011)
Macaques	1	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Abe et al.(2003b), Abe (2004)
	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Parkar et al.(2007)
	4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Parkar et al.(2010)
	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Scicluna et al.(2006)
	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al.(2004a)
	1	—	7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Present study
Vervet Monkey	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Abe et al.(2003b), Abe (2004)
	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Parkar et al.(2007)
De Brazza's Monkey	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Abe et al.(2003b), Abe (2004)
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al.(2004a)
	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Santin et al., 2011
Diana Monkey	2	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Present study
Leaf Monkey	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Abe et al.(2003b), Abe (2004)
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al.(2004a)
Japanese Monkey	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al.(1998)
	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al.(2003)
Woolly Monkey	2	2	1	—	—	—	—	3	—	—	—	—	—	—	—	—	—	—	Scicluna et al.(2006)
	—	1	3	—	—	—	—	14	—	—	—	—	—	—	—	—	—	—	Present study
Common Marmoset	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Present study
Lemurs	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Abe et al.(2003b), Abe (2004)
	—	—	—	2	—	—	—	2	—	2	—	—	—	—	—	—	—	—	Stensvold et al.(2009)
	3	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Parkar et al.(2007)
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	Present study
	1	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	1	Santin et al.(2011)
Allen Swamp Monkey	—	—	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Present study
Colobus	—	—	2	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	Present study
Ratas Monkey	1	—	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Present study
Siamang	2	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	Present study
Langur	—	—	2	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	Present study
Black Howler Monkey	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—	—	—	—	Present study
Sooty Mangabey	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Santin et al.(2011)
Hamlyn's Monkey	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	Santin et al.(2011)
Unidentified primates	—	1	5	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	Scicluna et al.(2006)
	1	—	2	—	—	—	—	1	—	—	—	—	—	—	—	—	1	1	Present study

Table 5.10 (continued)

Host	Blastocystis sp. Subtype (ST)																		Reference
	ST 1	ST 2	ST 3	ST 4	ST 5	ST 6	ST 7	ST 8	ST 9	ST 10	ST 11	ST 12	ST 13	ST 14	ST 15	ST 16	ST 17	Mix ST/ Bad seq	
Cattle	1	—	2	—	7	—	—	8	—	—	—	—	—	—	—	—	—	—	Abe et al. (2003c)
	1	—	1	—	6	—	—	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al. (2004a)
	—	—	—	—	3	—	—	—	—	22	—	—	—	—	—	—	—	—	Stensvold et al. (2009)
	—	—	—	—	—	—	—	—	—	7	—	—	—	—	—	—	—	—	Santín et al., 2011
	—	—	—	—	2	—	—	—	—	6	—	—	—	—	—	2	—	5	Present study
Cattle total	2	0	3	0	18	0	0	0	0	35	0	0	0	0	0	2	0	5	0
Camel	5	—	5	—	20	—	—	—	—	6	—	—	—	—	2	1	1	7	Present study
Horse	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Thathaisong et al. (2003)
Roe deer	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	Stensvold et al. (2009)
Mouse deer	—	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	Present study
Gazelle	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	Present study
Sheep	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	Stensvold et al. (2009)
Barbary sheep	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	Present study
Goat	—	—	1	—	—	—	1	—	—	1	—	—	—	—	—	—	—	1	Present study
Deer/mousedeer/gazelle/barbary sheep/goat/horse/camel total	5	0	7	0	20	0	1	0	0	10	0	0	2	0	2	1	1	9	0
Dog	1	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Parker et al. (2007)
Cat	—	—	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Eroglun et al. (2010)
Carnivores total	1	3	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pigs	3	—	1	—	8	—	—	—	—	—	—	—	—	—	—	—	—	—	Abe et al. (2003c)
	122	7	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	Ansue et al. (2003)
	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Navarro et al. (2008)
	20	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	Noel et al. (2003)
	—	—	—	—	16	—	—	—	—	—	—	—	—	—	—	—	—	—	Sciicluna et al. (2006)
	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	Thathaisong et al. (2003)
	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	Yan et al. (2007)
	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al. (1998)
	4	—	2	—	14	—	—	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al. (2003)
	—	—	3	—	17	—	—	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al. (2004a)
	—	—	—	—	7	—	—	—	—	—	—	—	—	—	—	—	—	—	Stensvold et al. (2009)
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Santín et al. (2011)
Pigs total	150	7	6	0	66	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Elephant	—	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—	Parker et al. (2010)
Quokka	—	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—	Parker et al. (2010)
Western grey kangaroo	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	—	Parker et al. (2010)
Quokka	—	—	—	—	—	—	—	—	—	—	—	2	—	—	—	—	—	—	Parker et al. (2010)
Opossum	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Parker et al. (2007)
Elephant/quokka/western grey kangaroo, quokka and Opossum total	0	0	0	1	0	0	0	0	0	0	4	6	2	0	0	0	0	0	0
Rat	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Noel et al. (2003)
	—	—	—	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Noel et al. (2005)
	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al. (1998)
Guinea pig	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Leipe et al. (1996)
	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Silberman et al. (1996)
Guinea	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Present study
Prudent total	0	0	0	7	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Non-primate mammals total	158	10	19	8	104	0	1	0	0	45	4	6	4	1	2	3	1	14	0
Quack	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	Noel et al. (2003)
Guinea	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	Abe (2004)
Chicken	—	1	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	Ansue et al. (2003)
	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	Noel et al. (2003)
	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al. (2003)
	2	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al. (2004a)
	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	Present study
	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Santín et al. (2011)
Guinea	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	Ansue et al. (2003)
	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al. (1998)
	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al. (2003)
	—	—	—	—	—	—	4	4	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al. (2004a)
Elephant	—	—	—	—	—	1	—	1	—	—	—	—	—	—	—	—	—	—	Abe et al. (2003a)
	1	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	Yoshikawa et al. (2004a)
Guinea	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	Abe et al. (2003a)
Partridge	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	Abe et al. (2003a)
Turkey	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	Hess et al. (2006)
	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	Noel et al. (2003)
Unidentified bird	2	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Eroglun et al. (2010)
Birds total	5	4	0	0	0	10	14	1	0	0	0	0	0	0	0	0	0	1	7
Mammals and birds total	207	43	57	11	113	10	16	30	0	47	4	6	4	1	2	3	5	21	10

## **The objective**

### **To investigate associations between *Blastocystis* subtypes and specific hosts**

In Denmark, ST10 was detected in 22 samples from cattle as well as from a lemur. In the USA ST10 was also detected from cattle. In Libya ST10 was found in various animals, six each from cow and camel and one each from goat and Barbary sheep. This shows that ST10 is present in different hosts and locations. In Libya most samples collected were from camel but only 47 of these samples were positive for seven *Blastocystis* STs (ST1, ST3, ST5, ST10, ST15, ST16 and ST17). In cattle, out of 15 samples three STs were detected (ST5, ST10 and ST14); if more samples from cattle were investigated more STs might be detected. However, it seems that ST10 is mostly restricted to artiodactyls.

ST7 is normally found in birds and considered avian subtype but it was also recently detected in non-human primate (Colobus monkey) in UK and in a goat in Libya. This suggests that host specificity of ST7 is not as strong as originally thought.

Another example is the rodent which is previously was found to carry ST4, the only nonhuman host for this ST until recently Stensvold *et al.* (2009) detected ST4 in a few monkeys as well. In this study a new ST was detected in a rodent called gundi that lives in North Africa, particularly in Libya. This was designed ST14 and is distant from other STs. This shows that rodents carry multiple STs.

In order to study the link between *Blastocystis* and specific host we have to collect more samples from multiple locations in the same country and from different geographic regions as well as from different animal hosts. Only doing this can help us to make strong conclusions

about *Blastocystis* ST and host specificity. Previously, conclusions were being made based on too few samples from too few hosts from too few locations.

**To investigate the possibility that *Blastocystis* infection is a zoonosis**

In order to investigate the possibility that *Blastocystis* infection is a zoonosis we have to collect faecal samples from animals and from the people who work with these animals, like zookeepers, farmers or animal handlers. It is also important to have another group of people which can be considered true control group who do not come in close contact with animals. For example in Libya, there are people considered to be camel handlers and they care for their camels by feeding, milking, even helping the camel to deliver its baby, so there is very close contact with the camel on a daily basis. By taken faecal samples from these camel handlers and their camels as well and comparing them with other groups in the same region that do not have any close contact with either camels or the camel handlers, we can compare the distribution of *Blastocystis* STs in all groups. This will reveal whether the camel handler STs are more similar to other humans or to their camels. However, it is possible that not all STs infect humans so this needs to be considered. It is also possible that identifying strains within STs will be necessary to confirm zoonotic infection. As already discussed persuading asymptomatic individuals to provide faecal samples is often not easy so it may prove difficult to obtain the samples necessary to complete this investigation.

## Chapter 6

# **Development of Multilocus Sequence Typing (MLST) for *Blastocystis* ST1**

## 6.1 Introduction

People have suggested zoonotic transmission of *Blastocystis* to humans occurs but we cannot prove a connection based on subtype alone. In addition, reports linking subtypes to symptoms are often contradicted by other studies. It is possible that strains within subtypes differ in ability to cause disease, or vary geographically.

In order to make a link or distinguish between strains of the same *Blastocystis* subtype, we need a method that detects genetic variation in non-ribosomal genes.

Sometimes a monkey carries the same *Blastocystis* subtype as found in zookeeper but it is not necessarily the same strain. For example, if a zookeeper has ST3 and a monkey they are looking after also has ST3 as well it does not mean that the ST3 in the monkey is the same strain as in zookeeper and therefore results from zoonotic transmission – ST3 is common in both hosts. So for us to prove that zoonotic transmission of *Blastocystis* occurs we do need a tool to allow us to distinguish between strains within *Blastocystis* STs and MLST is one of the best molecular techniques to carry out this differentiation.

Multilocus sequence typing (MLST) is a highly discriminatory and clear method of characterizing bacterial isolates that has now been successfully used in the characterisation of several eukaryotic species. MLST is based on the nucleotide sequences of internal fragments of housekeeping genes in which mutations are believed to be mainly neutral (Selander *et al.*, 1986). Different nucleotide sequences for each gene fragment are assigned allele numbers and the sequence type (SQT) of each isolate is defined by the combination of alleles present at each of up to seven distinct loci; the isolates that share the same SQT are believed to be members of the same clone (King *et al.*, 2002).



The aim of this study was to develop a MLST scheme for *Blastocystis* ST1 in order to understand the epidemiology and zoonotic potential of this parasite. Normally, nuclear genes are used for MLST loci but no genome sequence was available for *Blastocystis* at the start of this work, so we used the mitochondrion-like organelle (MLO) genome instead. For the same reason other methods of detecting strain variation, like microsatellites, were also not an option. Even now the only genome available is for ST7, a rare subtype in humans and too diverged to be useful for designing primers for the common subtypes. We have investigated ST1 as initial investigation showed variation in its MLO genome sequence and also because the whole MLO genome sequence is available in GenBank. It is also a common ST in humans. The MLST scheme is based on nucleotide sequences of seven genome fragments and was used to evaluate 64 isolates from various hosts and geographical sources. This is the first report of an MLST scheme for *Blastocystis*.

## **6.2 Material and Method**

### **6.2.1 *Blastocystis* isolates**

A total of 64 *Blastocystis* isolates that had previously been identified as ST1 by barcoding were collected from different hosts and geographical locations. Thirty-nine of these isolates were eventually used in this study and their origins are shown in Table 6.1. The other 25 isolates did not give complete results either because of problems with amplification or sequencing reactions for one or more loci.

DNA samples from Denmark were provided by Dr Rune Stensvold (SSI, Copenhagen). These were extracted directly from faeces.

**Table 6.1** *Blastocystis* ST1 isolates from different hosts and locations

Host	UK	Denmark	Libya	USA	Total
Unselected	14	4	3	1	22
IBS	7	0	0	0	7
NHP	7	0	0	0	7
Zoo keepers	3	0	0	0	3
Total	31	4	3	1	39

**6.2.2     *Blastocystis* culture and DNA extraction**

These methods were as described previously. See chapter 2, section 2.1-2.7 for details.

**6.2.3     Selection of gene fragments for MLST scheme**

The whole mitochondrion-like organelle (MLO) genome sequence for *Blastocystis* NandII strain (ST1) was available in GenBank (Accession number EF494740; Fig. 6.1). Initially, 17 gene fragments were chosen for a pilot MLST study of 8 *Blastocystis* ST1 isolates. Loci that did not give reliable amplification or showed no variation or did not show good sequence results were eliminated as targets. Results from these pilot sequences were used to establish a final group of seven gene fragments that gave the highest discrimination for MLST analysis. Each region showed at least two polymorphic sites in the pilot study. Primers were designed to amplify gene fragments of 400-600 bp and are described in Table 6.2, with the corresponding genome location given. Two regions of the nad1 gene were used as were part of nad7, nad5 and nad9. Two loci contain parts of more than one gene. Locus 2 contains part of rps8 and rp16 while locus 7 contains part of Orf143 and rps12 and in between is a complete tRNA Trp gene.

Perez-Brocal (unpublished) found that most protein-coding genes in the MLO genome of *Blastocystis* are under purifying selection. Purifying selection removes deleterious alleles

from the population and so this affects calculations of divergence times because it leads to an underestimation of the number of mutations that have occurred. The effect this will have on MLST is that the diversity is likely to be lower than if there was no purifying selection and there are likely to be fewer non-synonymous substitutions. However, even though most genes in the *Blastocystis* MLO are under purifying selection this has no impact on the usefulness of the MLST method that has been developed, because regions of a representative set of genes have been used (approximately 25% of the total number of protein-coding genes in the genome (27))

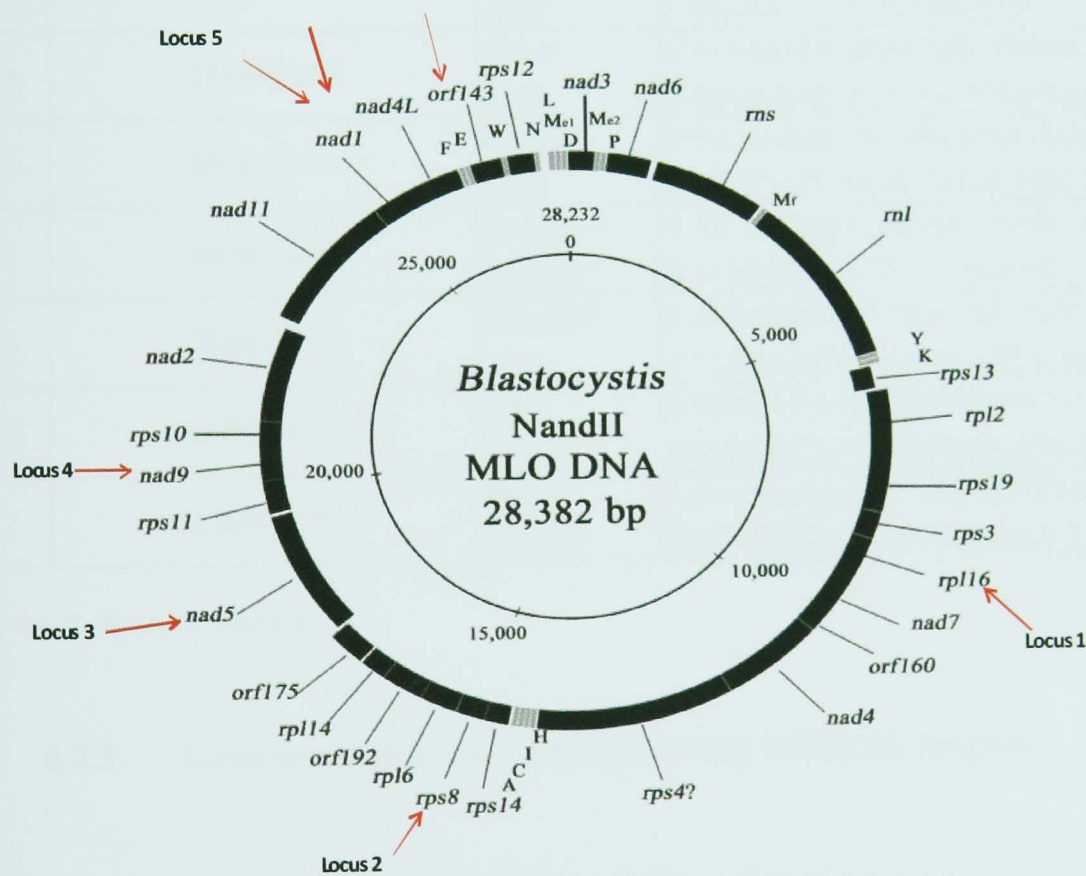


Fig 6.1. The gene map of the MLO genome of *Blastocystis* Nand II (EF494740) and target loci used in the MLST study. Arrows indicate the location of the regions amplified.

6.2.4 Primer design

Most of the primers sets used initially in this study were designed for the original MLO genome sequencing by Perez-Brocal and Clark (2008). Additional/alternative oligonucleotide primers for PCR at the seven MLST–determining loci were designed by eye, or using the Gene Runner software version 3.05, from the MLO genome sequence of *Blastocystis* Nand II.

Table 6.2 Primers used for PCR amplification and sequencing of *Blastocystis* ST1

Locus	Location of the locus in Nand II	Primer name	Sequence (5'-3')
1	8711-9190	22BF 23BR	5' GTGAAATTGTTCAAAAAGCAG 3' 5' GGGAATGTTTTCTTAAATCC 3'
2	15459-15842	NS14F NL6R	5' ACAAATATGGTATAGCTCGTACTG 3' 5' AAAGTTAGTTTGAAATTAATAATACC 3'
3	19459-19733	Nnad5F 56BR	5' TGCAAAGACTGTAGAATAATCAAC 3' 5' AGGTTCTTTTAAATTATATTGGG 3'
4	20797-21099	Nnad91-F Snad9-R*	5' ATCAATTGCACACATATCGGAC 3' 5' ATGGGAAATCTTTCTWARMGG 3'
5	25306-25621	Nnad11F 71BR	5' ATTAGTATGTCTTCTCAAATTATGTC 3' 5' CCAGAAATAATAATACCATATATAGC 3'
6	25898-26134	72BF Snad1R	5' CTTATGAACTTTCTATTGGTGC 3' 5' CCTATTCTCATTAATTGATCATAAC 3'
7	27394-27691	78B2F NRS12R	5' AAGTCAAGAAATTTATAATTCATTGTTTC 3' 5' GCTATATTCCTGGTATTGGTCA 3'

\*W=A/T, R=A/G, M=A/C

6.2.5 Gene amplification and sequencing of MLST targets

For each locus, primers were designed to have a similar melting temperature and loci were found to be successfully amplified by PCR. The details of seven primers sets used in PCR and sequencing reactions are listed in Table 6.2.

The total reaction volume for DNA amplification was 20 µl. DNA amplification was performed using thermocycler G-storm Model GS-1 (Gene Technologies Corp) with a programme consisting of a 2 minutes denaturation step at 94 °C, followed by 30 cycles of 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 2 minutes, and ending with a final extension for 2 minutes at 72 °C. Another protocol was also tested (as some isolates did not

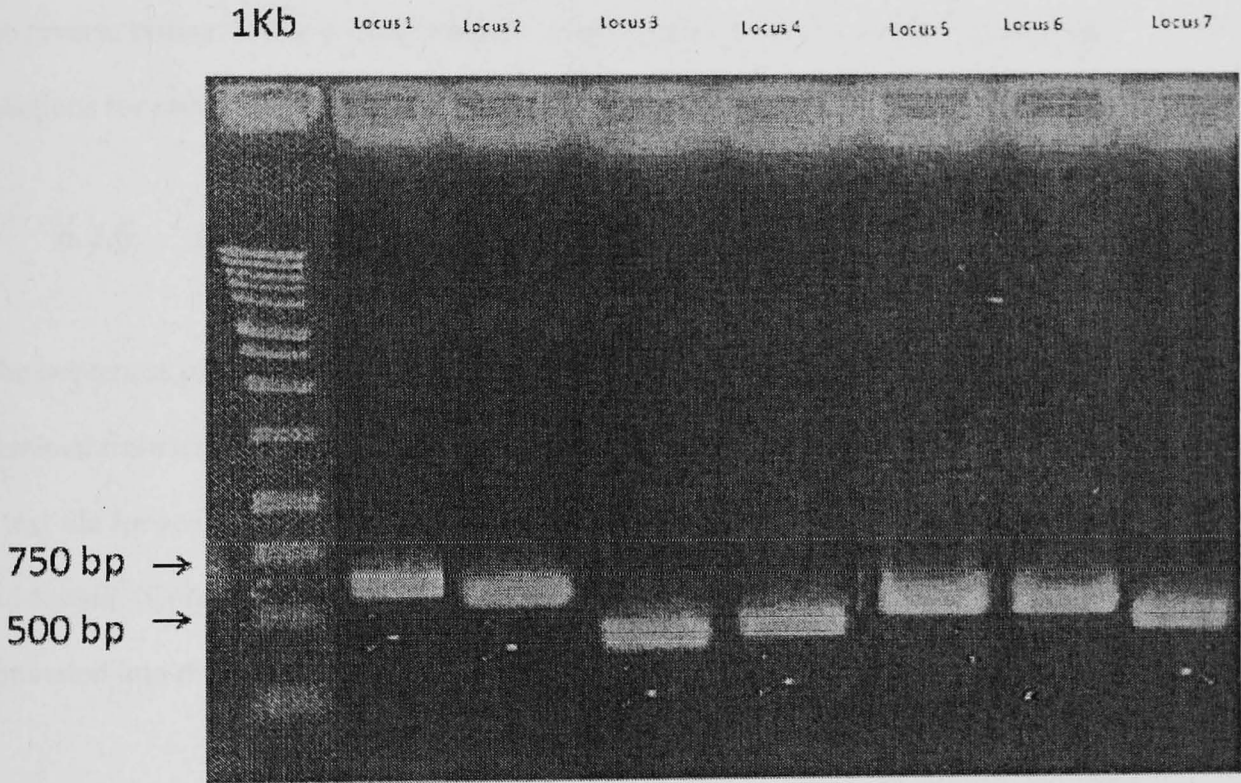


Fig 6.2. PCR result for MLST of seven loci

Locus 1= 613 bp, locus 2 = 596 bp, locus 3= 397 bp, locus 4= 407 bp, locus 5 & 6 = 455 bp and locus 7 = 431 bp. DNA ladder 1kb.

amplify with the previous programme) with an initial denaturation at 94 °C for 2 minutes, followed by 10 cycles of touch-down PCR (denaturation at 94 °C for 30 seconds, annealing at 60 °C, decreasing by 0.5 °C per cycle, and extension at 68 °C for 4 minutes), then 20 cycles with denaturation at 94 °C for 30 seconds, annealing at 56 °C for 30 seconds, and elongation at 68 °C for for 4 minutes (Fig 6.2).

The GeneJET PCR purification kit (Fermentas, York, UK) was used to purify the product. The procedure was carried out as in Chapter 2 except 20 µl of sterile deionized water were used to elute the DNA instead of 50 µl of elution buffer (resulting in more concentration of the DNA preparation). The PCR products were sequenced on both DNA strands. The same sequencing reaction protocol was used as in chapter 2; for each PCR amplified locus two sequencing reactions were carried out separately, one with the forward primer and one with the reverse primer. Primers used in MLST amplification were also used in sequencing reactions for each locus with 1:10 dilution.

#### **6.2.6 MLST analysis**

The sequences of each of the seven selected 280-480 bp double stranded regions were obtained from a subset of 39 *Blastocystis* ST1 isolates. The edited sequences were saved into a text file for analysis. For each locus analysed, a multiple alignment was manually created in Multalin (Corpet, 1988) from all those isolates sequenced. The sequence file was later converted into different formats for analysis using the BioEdit and MEGA programmes.

For each locus, different sequences were allocated distinct allele numbers. This resulted in a 7-digit allelic profile for each isolate. Each unique allelic profile was manually assigned a sequence type (SQT) number. Isolates with the same allelic profile were assigned to the same SQT, and are considered to be members of a single clone. The construction of a dendrogram (based on concatenated DNA sequences of all loci) by the Neighbour-Joining method was also performed in MEGA 4.0; see Chapter 2 sections 2.8 for details.

**Table 6.3** Allelic diversity among 39 *Blastocystis* ST1 isolates

locus	Amplicon size (bp)	Sized of sequenced region (bp)	No of alleles	No of polymorphic sites	% of polymorphic sites	Mean %G+C content
1	613	480	7	10	2.08%	25.57%
2	596	388	12	17	4.38%	16.57%
3	397	276	8	7	2.53%	25.04%
4	407	303	10	9	2.97%	20.25%
5	455	316	4	3	0.94%	27.15%
6	455	280	10	10	3.57%	27.22%
7	431	300	10	9	3%	20.76%

**6.3 Results**

For all 39 isolates, PCR products of the expected size were obtained for the seven loci. Several new primers (23BF, 22BF, 56BF, 71BR, 72BF and 78B2F) were designed for use in this work as shown in Table 6.2.

**6.3.1 Allelic variation in *Blastocystis* ST1**

The sequence diversity within the *Blastocystis* ST1 genes was low but was sufficient to distinguish 4-12 alleles for each selected locus (Table 6.3). The number of isolates with each allele for all seven loci is shown in Table 6.4. The locus with the most alleles was locus 2 (12 alleles) while the locus with the fewest alleles was locus 5 (4 alleles)

A total of 32 SQTs were recognised within the 39 *Blastocystis* isolates in this study (see Table 6.4). This gives a discriminatory index of 0.98. A discriminatory index of > 0.9 is required for a MLST scheme to be useful (Hunter and Fraser, 1989). The relationship between SQTs was displayed as a phylogenetic tree created from the concatenated sequences (Fig.6.3).

### 6.3.2 MLST analysis of *Blastocystis* ST1

The majority of SQTs (28) were represented by single isolates. Among SQTs shared by more than one isolate, the most frequently encountered were SQT10 (4 isolates), SQT2 (3 isolates), SQT15 and SQT16 (2 isolates each) (Table 6.4).

The phylogenetic tree shows that ST1 can be divided into four clades (Fig 6.2). Clade 1 has most of the isolates with a variety of different hosts represented and a wide geographical distribution. Clade 2 has two non-human primates, both siamangs collected from the same zoo (Monkey World). Clade 3 has two isolates, one a primate keeper from the UK and the other an unselected individual from Denmark. Clade 4 consists mainly of unselected samples plus one each from an IBS patient and a zoo keeper; in this clade isolates came from four different geographical places (Libya, UK, Denmark and USA).



**Table 6.4** Result of the MLST analysis of the isolates of *Blastocystis* subtype 1

Isolates	SQTs	Allelic profile							Geographic origin	Host
MA		1	2	3	4	5	6	7		
3	1	1	1	1	1	1	1	1	UK	IBS
4	2	1	2	1	1	1	2	2	UK	Diana Monkey
9	3	1	2	2	1	1	2	3	UK	Unselected
16	4	1	3	1	1	1	2	2	UK	Mandrill
27	5	1	4	1	1	1	2	4	Denmark	Unselected
29	6	1	2	2	1	1	2	5	Denmark	Unselected
39	7	1	2	2	2	1	2	2	UK	Unselected
48	8	1	5	1	3	2	1	2	UK	Unselected
53	9	2	6	3	4	1	3	2	UK	Zoo keeper
55	10	1	1	1	1	1	1	2	UK	Unselected
57	2	1	2	1	1	1	2	2	UK	Zoo keeper
70	11	3	7	3	5	1	3	6	Denmark	Unselected
71	12	4	8	4	4	3	3	7	UK	Siamang
73	13	5	9	5	4	2	3	6	Denmark	Unselected
76	14	5	9	1	4	2	3	8	UK	Zoo keeper
90	15	1	2	6	1	1	2	2	UK	IBS
95	15	1	2	6	1	1	2	2	UK	IBS
111	16	5	10	1	4	2	3	2	UK	Unselected
117	17	5	9	1	6	2	3	2	UK	IBS
138	10	1	1	1	1	1	1	2	UK	IBS
143	18	4	8	4	4	3	3	2	UK	Siamang
166	19	1	2	2	7	1	2	2	UK	Unselected
170	20	6	2	3	1	1	4	2	UK	Unselected
178	16	5	10	1	4	2	3	2	UK	Unselected
200	21	1	1	1	8	4	1	2	UK	IBS
206	22	5	9	7	4	2	3	6	UK	Unselected
225	23	1	1	3	4	1	1	2	UK	IBS
227	2	1	2	1	1	1	2	2	UK	Unselected
258	24	1	11	1	1	1	5	2	UK	Diana Monkey
260	25	1	2	8	1	1	6	4	UK	Orangutan
276	10	1	1	1	1	1	1	2	UK	Mandrill
289	26	1	11	1	1	1	2	9	UK	Unselected
298	27	5	12	1	4	2	3	2	UK	Unselected
402	28	1	11	1	9	1	7	2	UK	Unselected
409	10	1	1	1	1	1	1	2	UK	Unselected
LHR17	29	5	9	1	4	2	8	2	Libya	Unselected
LHR24	30	7	9	1	10	2	9	10	Libya	Unselected
LHR7	31	5	9	1	4	2	3	6	Libya	Unselected
NandII	32	5	9	1	4	2	10	2	USA	Unselected

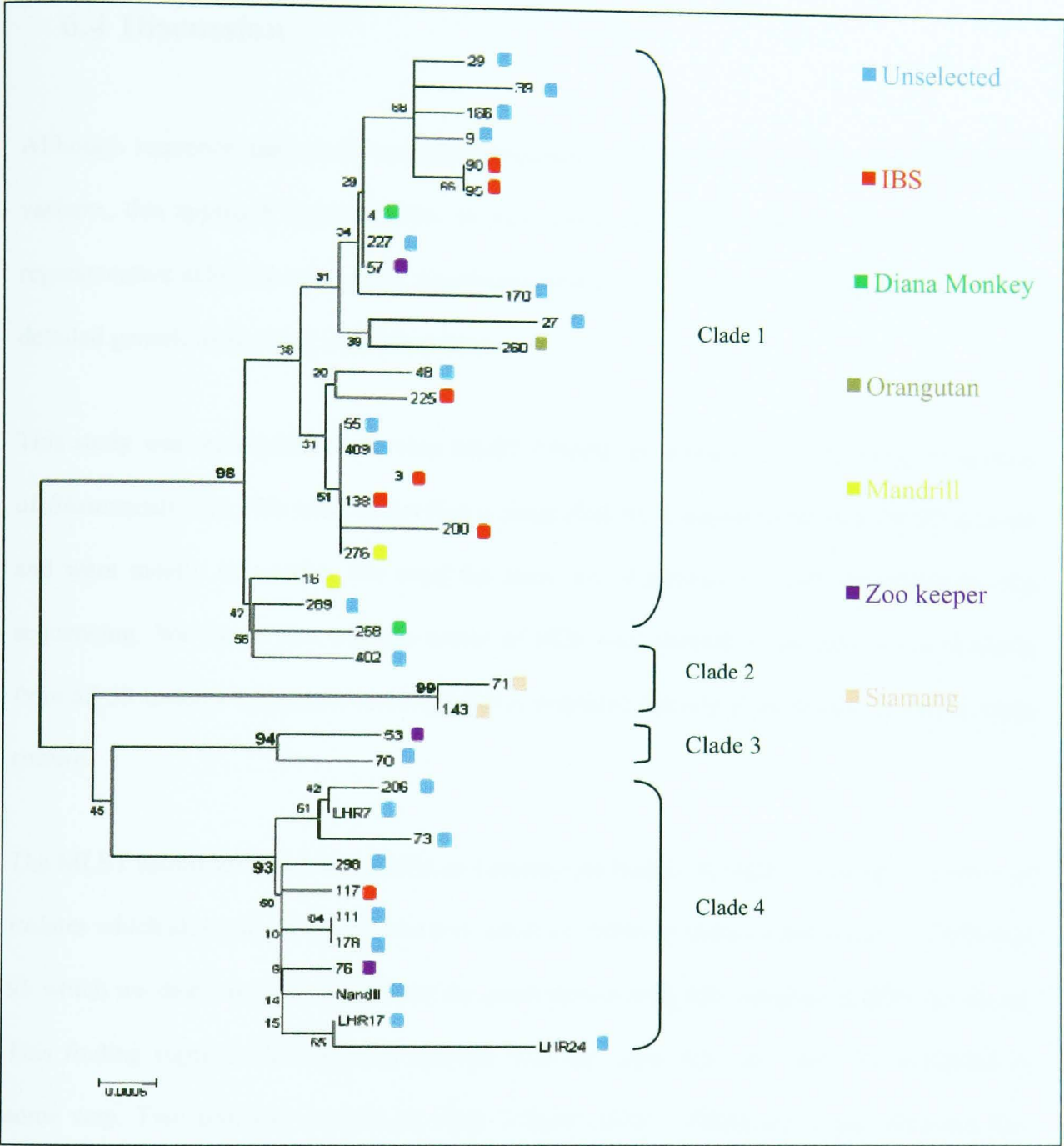


Fig 6.3. Neighbor-joining phylogenetic tree of 39 isolates of *Blastocystis* ST1 based on the concatenated sequences of seven MLST loci. Numbers represent bootstrap support for the branch points. Bootstrap values of over 90% are shown in bold and a larger font size.

## 6.4 Discussion

Although sequence analysis of complete genomes is the only way to distinguish all genetic variants, this approach is not feasible in most cases and is not necessary. Sequencing of a representative selection of regions distributed over the *Blastocystis* genome can also allow detailed genetic analysis of individual strains.

This study was undertaken to develop MLST scheme for *Blastocystis* ST1 using 39 isolates of *Blastocystis* ST1. We tried to develop a simplified PCR approach for this MLST scheme and were mostly successful. We used the same set of primers for both amplification and sequencing. We found that a single round of PCR was suitable to amplify seven products from all 39 isolates examined, including DNA extracted directly from faeces as well as from culture.

The MLST results revealed four clades as I mentioned before. In clade 1 there are 3 groups of isolates which showed same SQT and they are from different hosts except isolates MA90 and 95 which we discovered later are from the same person with IBS sampled at different times. This finding supports the idea that samples with the same SQT are likely to be related in some way. Two siamang samples in clade 2 have closely related SQTs and although this shows some evidence of host specificity of clades within a ST, the number of samples is too small to make a significant conclusion.

It is clear from this that this MLST can allow us to make a strong link between infections as the discriminatory power is high enough to differentiate between most strains. MLST can be used as a tool to differentiate between *Blastocystis* ST1 strains within the same host species or to study the zoonotic transmission by making a link between the same *Blastocystis* ST in

different hosts. In the strains studies so far no clear evidence of zoonotic transmission or host specificity was found. Even though a zookeeper and a Diana monkey had the same SQT they are from different zoos. Yoshikawa *et al.* (2009) reported that the prevalence of ST2 in human and monkey isolates offered molecular evidence of the potential transmission of *Blastocystis* between these hosts. I think that without using a technique like MLST to distinguish between strains of *Blastocystis* within a subtype it is difficult to accept this conclusion.

In summary, our data suggest strongly that the seven loci chosen are a suitable basis for a MLST scheme, as we could amplify and sequence from isolates obtained from a wide variety of sources and detect sufficient diversity to provide a high degree of resolution. In addition MLST gives high reproducibility and has the potential for sharing of data through the internet, as has been done for MLST schemes in bacteria.

## **The objective**

### **To develop a MLST method that distinguishes strains within *Blastocystis* subtypes**

We have developed MLST for *Blastocystis* ST1 and the discriminatory power is more than 0.9. With this method at least 4 clades were detected within ST1 and these clades did not show any link to pathogenicity, host specificity or geographical distribution. However, it can be a basis for study to determine the transmission of *Blastocystis*. Additional sampling is needed to determine whether the few clades detected cover the range of diversity within ST1. It is also important to test whether these loci can be used to study, ST1 in non-primate hosts. Because of the divergence between *Blastocystis* STs it will be necessary to develop a separate

MLST for each one – the MLO genome available in genBank differ by almost 25%.

However, the successful development of MLST for ST1 shows that it should be possible.

## **Chapter 7**

### **Final discussion**

When I started my study there were only nine STs of *Blastocystis* in birds and mammals but not many hosts been sampled. There was limited information on *Blastocystis* ST distribution. No clear evidence of zoonotic transmission and no clear link between pathogenicity and *Blastocystis* STs had been published.

In this study a major finding was the discovery of four new *Blastocystis* STs from different hosts and also four new hosts for *Blastocystis* from UK and Libya (see Chapter 5). The more we do sampling we are likely to get many new ST and hosts as well.

My survey showed that ST1 was the most common ST in Libya and this is quite different from most other parts of the world where ST3 is the most common ST. It will be very useful to collect samples from different regions of Libya to compare the distribution of *Blastocystis* STs with these results, to see whether it will show the same pattern or not. In Egypt two studies from different regions revealed different *Blastocystis* ST distribution. We need to investigate whether it is going to be the same situation with Libya.

To investigate the link between ST and disease, we need a clear diagnosis of symptomatic individuals infected with *Blastocystis* by excluding all bacteria, virus, and other parasitic diseases as well as food allergy, ulcerative colitis and Crohn's disease to eliminate any bias in the results and make any conclusions stronger. Also important is having a good asymptomatic control group that matches the symptomatic group as closely as possible. The fact I did not have such a group made interpretation of my results more difficult.

To study the possible link of *Blastocystis* ST with IBS I think we need to be certain of the ST identification by following the same techniques so that we avoid any errors. For instance, Yakoob *et al.* (2010a) compared samples from IBS and an unselected control group using

sequence –tagged site (STS) analysis. He found high ST1 in IBS while ST3 was the second most common ST, but in the unselected group ST3 was the highest. In our study ST3 was high in both IBS and unselected group but ST4 was higher in IBS compared to unselected group. Comparing the Yakoob *et al.* (2010a) result with *Blastocystis* distribution from different countries (Table 3.2 chapter 3) only Iran and Libya showed high ST1. Since different methods were used to identify subtypes in this study and in Yakoob's study we need to be sure that they both give the same results with the same samples.

Quite a number of animals in this study showed mixed ST infection compared to the frequency in human samples and to overcome this problem Stensvold *et al.* (2009) suggested using ST-specific primers which can be developed for PCR studies together with the genus-specific primers that are already in use. The problem with this procedure is that it will lead to extra cost, as we now have 17 STs, and ST-specific primers will not have the ability to detect new STs.

Development of MLST for the most common *Blastocystis* ST1-ST4 is needed to better understand zoonotic transmission, pathogenicity and geographical distribution of this parasite. Already, we can study zoonotic transmission using MLST for *Blastocystis* ST1 by collecting samples from animals and their keepers to investigate the link between strains within this subtype. However we do not know whether the same results will be obtained for all subtypes. The genetic distance between subtypes means that a separate MLST needs to be developed for each ST, which means the ST will have to be identified before MLST can be performed. MLST can also be used to study geographical distribution of *Blastocystis* by comparing strains within a subtype in samples from different geographical areas. It is possible that the amount of diversity will vary between locations as well as different SQTs being found.



The objectives of this study were:

To compare the *Blastocystis* subtype distribution between the UK and Libya

Our result showed that there is a difference in *Blastocystis* ST distribution between the UK and Libya. ST3 was the most common ST in UK while ST1 was the most frequent ST found in Libya. No ST4 was detected in Libya but in the UK it is the second most common ST.

To investigate *Blastocystis* subtypes in IBS patients

This study showed that ST4 is more common in IBS compared to an unselected group and there is an indication that ST4 in UK is associated with IBS even though there was no significant difference.

To investigate associations between *Blastocystis* subtypes and specific hosts.

In our study different STs were detected in the same host but also different hosts showed the same ST. For example ST13 was detected in a quokka in Australia and I have detected the same ST in mouse deer. Some STs showed more host specificity than other STs like ST14 and ST15 which were identified only in gundi and camel respectively.

To investigate the possibility that *Blastocystis* infection is a zoonosis.

Some STs may show some evidence of zoonotic transmission like ST1, which was detected in camel and identified in Libyan humans as well. Also ST7 was detected in humans in Libya and in a chicken. Similarly in UK ST8 was detected in zoo keepers and also showed a high frequency in non-human primates. However the most common STs in Libyan animals are not found in human population.

To develop a MLST method that distinguishes strains within *Blastocystis* subtypes.

Concatenated sequences of seven MLST loci for 39 isolates of *Blastocystis* ST1 illustrated four clades in the phylogenetic tree and we were able to differentiate most strains within *Blastocystis* ST1. Its usefulness was shown by two isolates that have an identical SQT, and were later found to be samples from the same person at different times.

Overall, the original objective of this project have been met to varying degrees, and I certainly believe I have contributed to the aim of increasing our understanding of genetic diversity of *Blastocystis*

## **Future work**

### **Human studies**

#### **Distribution of *Blastocystis* STs in human:**

My results analysing *Blastocystis* ST distribution in Libya and the UK showed a significantly different prevalence even though the samples from Libya were low in number and collected from only one region, Sebha. Similarly the UK the samples were received from only one source (the Diagnostic Parasitology Laboratory, LSHTM)

To confirm whether there is really variation in the distribution of *Blastocystis* STs between Libya and UK populations a more complete survey is needed.

#### **Libyan samples**

The samples used for our study were collected from the Central Medical Laboratory in Sebha and we do not know the clinical background of these patients. Many are likely to be samples submitted for routine screening but some samples might be from symptomatic patients as well.

154 samples were used to identify *Blastocystis* in Sebha, 43 samples were positive by culture and in only 38 samples were *Blastocystis* STs identified. To improve our understanding, we should obtain the same number of samples from asymptomatic patients in both the Tripoli and Benghazi regions to obtain clear view of the overall *Blastocystis* ST distribution in the Libya population. We have already contacted some clinics in both regions who agreed to

collect samples from asymptomatic patients visiting these clinics for reasons other than gastrointestinal symptoms, for example routine health checks.

### **UK samples**

Previously, all UK samples have been received from the Diagnostic Parasitology Laboratory at LSHTM and the problem with these samples is that we do not have access to the clinical history of these patients. Because of this these samples could be from IBS patients or symptomatic patients with causes other than IBS. It is unlikely that many are from asymptomatic individuals, because samples are referred for diagnosis by GPs or IBS clinics.

As asymptomatic patients may be infected with STs that are different from those with symptoms, asymptomatic patients should be clearly identified in both countries. Both gender and age information should be collected as well to give more information on the epidemiology of *Blastocystis* STs in these populations.

By collecting samples randomly from Tripoli (North West of Libya) and from Benghazi (North East of Libya), which are different in climate from Sebha (South West of Libya), and joining them with the data which I already have from Sebha this will give a more complete picture of *Blastocystis* prevalence and STs in Libya as well as showing any influence of climate and geography. In the UK, samples still need to be collected randomly from different regions. All samples received from the Diagnostic Laboratory at LSHTM came from England and at the present no sources from other regions have been identified, but in the future this could be organized.

Together these new samples will give the real prevalence and distribution of *Blastocystis* STs in Libya and UK populations.

### ***Blastocystis* STs and IBS**

Results showed the frequency of ST4 was much higher in IBS clinic patients than in unselected GP patients, although the difference was not statistically significant. Because unselected GP random samples received through the Diagnostic Laboratory at LSHTM may include patients with IBS and some samples from IBS clinics may have come from patients eventually found not to have IBS, to avoid any bias in the results in future we need to confirm the correct diagnosis (IBS, asymptomatic or symptomatic patients with intestinal symptoms other than IBS) in order to address any link between *Blastocystis* subtypes and IBS. This will require ethical approval and cooperation of doctors submitting the samples, but should be possible. The most difficult aspect is likely to be obtaining a good control group for comparison to the IBS patients. This will require further investigation.

## **Animal studies**

### **Zoonotic transmission of *Blastocystis* infections**

Our results showed that ST1 and ST3 are the most common in humans and are found in some animals as well, including camels and goats. We do not know whether these STs are transmitted from animals to humans or the other way around, or in fact whether there is no cross-transmission.

To investigate the possible zoonotic transmission of these *Blastocystis* STs we can use MLST to see if there is any link between the sequence types in human and livestock. Libyan people as a group have more contact with sheep than goats due to a religious event where a hundred thousand sheep are slaughtered on one day every year. I propose to collect sheep samples from

different regions in Libya and from sheep handlers, as well from humans who do not have any contact with sheep, and hopefully sheep will be infected with ST1 and ST3. Sheep in other countries have been found to be infected with ST3. We can use the samples to investigate whether there is any cross transmission between sheep and human. A MLST scheme for ST1 already exist from my work and a MLST scheme for ST3 is being developed by Dr R.Stensvold (SSI, Copenhagen) so the tools will be available for this investigation.

***Blastocystis* subtypes and specific hosts:**

By comparing tables 3.2 & 5.10, showing the distribution of both human and animal *Blastocystis* subtypes worldwide, we noted that ST5 and ST10 are common in certain animals but very rarely found in humans. So whether these STs are specific to animals needs further investigation by screening more samples in cow, gazelle, sheep, gundi, goat and Barbary sheep and in a greater variety of animal hosts. For example the one gazelle sample available showed *Blastocystis* which was a mix of subtypes, therefore screening more samples from Gazelle is needed to identify the subtypes, or even new subtypes, that can be found in this host. As mentioned before cow, camel and goat can be infected with ST1 and ST3, which are also common in human, but whether these subtypes also found in other animals as well we do not know. Screening animal samples from different locations and including human samples from the same location as well is necessary. Screening individuals who work with the animals would be particularly informative, but may be difficult due to cultural issues with handling of faeces. I hope that by performing the additional studies described in this section that a clear picture of *Blastocystis* epidemiology will be obtained.

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